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INACTIVATION OF THERMODURIC SPOREFORMERS AND SPORES IN SKIM MILK BY CONTINUOUS ULTRASONICATION AND HYDRODYNAMIC CAVITATION IN COMBINATION WITH THERMAL TREATMENTS

BY

DIKSHI BAWA

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Science

Specialization in Dairy Science

South Dakota State University

2016

INACTIVATION OF THERMODURIC SPOREFORMERS AND SPORES IN SKIM MILK BY CONTINUOUS ULTRASONICATION AND HYDRODYNAMIC CAVITATION IN COMBINATION WITH THERMAL TREATMENTS

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

(Sanjeev Anand, Professor)
Thesis Advisor Date

(Vikram V. Mistry, Professor)
Head, Dairy Science Department Date

Dean, Graduate School Date

I am dedicating this thesis to God for giving me strength to pursue my dreams and my late grandmother for being my inspiration

&

To my parents and my husband for being my support system throughout this time

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ABBREVIATIONS

ATCC	American Type Culture Collection center
ANOVA	Analysis of variance
BHI	Brain Heart Infusion
$CaCl_2$	Calcium Chloride
CFU	Colony Forming Unit
CIP	Cleaning in place
DNA	Deoxyribonucleic Acid
ESL	Extended Shelf Life
FDA	Food and Drug Administration
g	gram
HC	Hydrodynamic Cavitation
HPP	High Pressure Processing
HTST	High Temperature Short Time
kPa	Kilopascal
L/h	Liter/ hour
Log	Log_{10}
LTLT	Low Temperature Long Time
MF	Microfiltration
mL	milli Liter
NaCl	Sodium Chloride

PBS	Phosphate buffer saline
PEF	Pulsed electric field
RAPD	Random Amplified Polymorphic DNA
SE	Standard Error
SAS	Statistical Analysis Software
SEM	Scanning Electron Microscopy
UHT	Ultra-high Temperature
US	Ultrasonication

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ABSTRACT

INACTIVATION OF THERMODURIC SPOREFORMERS AND SPORES IN SKIM MILK BY CONTINUOUS ULTRASONICATION AND HYDRODYNAMIC CAVITATION IN COMBINATION WITH THERMAL TREATMENTS

DIKSHI BAWA

2016

Studies were conducted to determine the effectiveness of ultrasonication and hydrodynamic cavitation, in a continuous mode, alone and in combination with thermal treatments, on heat resistant aerobic sporeformers and spores in skim milk, and skim milk concentrate.

The first study was conducted to investigate the effectiveness of a continuous ultrasonication process to reduce thermoduric *Bacillus coagulans* cells in skim milk. A 25% reduction was obtained by lab pasteurization alone, whereas ultrasonication alone resulted in an inactivation of 92% after 12 passes (80 sec exposure time per pass). Ultrasonication when combined with pasteurization resulted in further increased inactivation to 99.98%. It can thus be concluded that a continuous ultrasonication process followed by pasteurization is effective to inactivate thermoduric *Bacillus coagulans* cells.

The second study was conducted to investigate the effectiveness of a continuous hydrodynamic cavitation process to reduce thermoduric spore forming

bacilli in skim milk. The study also evaluated its impact on skim milk concentrate, and the final sporeformer counts of skim milk powder, in a simulated pilot scale skim milk manufacturing process. The results revealed that 6 pass cavitation effect alone and in combination with pasteurization was very effective in inactivating thermally resistant vegetative cells of *Bacillus coagulans* by 99.963% and 99.996% respectively. A 99.66% reduction of *Bacillus coagulans* cells was found in non-fat dry milk when made from cavitated milk, and upto a 99.65% reduction of total count was found in skim milk concentrate after cavitation treatment.

The third study was conducted to investigate the effectiveness of a continuous hydrodynamic cavitation process to reduce thermoduric spores of *Bacillus licheniformis* in skim milk. Heat treatment alone did not result in any inactivation of the spores. Cavitation with holding at 30ºC for 3 hours and heating at 85°C for 15 min resulted in an inactivation of 97.59%. Another treatment with preheating at 65°C along with cavitation and holding at 30ºC for 3 hours followed by heat treatment at 80°C for 1 min resulted in inactivation of 99.29%.

Overall, both ultrasonication and hydrodynamic cavitation when combined with heat treatment were found more effective in reduction of thermally resistant bacilli in skim milk.

INTRODUCTION

Milk is one of the richest sources of nutrition and this makes it one of the most suitable food for human consumption. It is composed of various essential components useful for human body. This makes dairy a lucrative business and increases a need to have extended shelf life dairy foods that are safe for consumption. Milk is, therefore, converted into various dairy ingredients such as non-fat dry milk, concentrated milk, whey powder, lactose etc., and in to various consumer products such as cheese, ice-cream, yogurts, dairy creamers etc. Milk and dairy products also provides favorable conditions for food spoilage bacteria to multiply, and their metabolic activities can impact product quality. Sporeforming bacteria of genera *Bacillus, Anoxybacillus, Paenibacillus,* and *Geobacillus* are some of the most prominent contaminants of raw milk. These bacteria can withstand thermal treatments and can get carried over to the final product impacting the quality and shelf life of that product as well. Due to this reason, controlling microbial quality of milk and milk products is of great significance in providing wholesome dairy products to the consumers.

Among spore formers, *Bacillus* and its related species are some of the major Gram-positive aerobic microorganisms, which are a concern for dairy industry. Species such as *B. anthracis, B. cereus,* and *B. licheniformis* are known to cause illnesses if they are entered in human body (Scheldeman et al., 2006, Burgess et al., 2010a). They also cause various spoilages in dairy products during storage. Spoilage is related to various flavor, taste, smell and textural defects such as bitter, unclean,

rancid, acidic or sour taste, off-notes, curdling or thickening of product and ropy texture etc. (Meer et al., 1991, Berkeley et al., 2008). These bacilli are capable to grow at a wide range of temperature and can produce enzymes that result in lipolysis of fat and proteolysis of protein present in milk (Robinson, 2002). One of the biggest problems with these organisms is their ability to adhere to stainless steel equipment surfaces in dairy plants and form biofilms resulting in in-process contamination (Scheldeman et al., 2006, Hassan et al., 2010). The spore formers do not get killed and then they get converted to spores under adverse and stressful conditions such as insufficient nutrition or inappropriate growth environment. The converted spores resist severe heat treatments, radiations, chemicals and high pressure, which make them capable to survive under unfavorable conditions (Setlow, 2006b, Henriques and Moran, 2007, Burgess et al., 2010a). In Dairy plants, they ideally grow in temperature zone of 40 to 65°C, mainly in regeneration sections of heat exchangers, preheaters, pasteurizers and evaporators, and result in high numbers in the final product (Scott et al., 2007b).

Thermal treatment such as pasteurization and UHT treatment are traditionally used over decades to ensure quality and safety of milk. These processes are capable to kill most of the spoilage and pathogenic bacteria, but they show a limited effectiveness on thermoduric spore formers and their spores. Processing at a very high temperature of 150°C also been reported to be insufficient to achieve complete inactivation of some spores (Lewis and Deeth, 2009b). At the same time, these treatments cause adverse impact on sensory attributes such as color and flavor, and

also have the ability to alter nutritional composition of milk due to significant nutritional losses. This has prompted researchers, and food manufactures to identify alternate ways of manufacturing high quality, nutritious, and safe foods. Some of the alternate methods include studying the effectiveness of various non-thermal techniques and combining them with existing heat treatments. The possible hypothesis behind doing so is that the non-thermal treatments can potentially weaken the cell membrane, which can further be damaged by heat treatment in the following steps. Techniques such as cavitation, bactofugation, microfiltration, pulse electric field, non-thermal plasma, high pressure processing, high pressure homogenization etc. have been tested for their effectiveness in bacterial reduction in milk and other dairy products (Tahiri et al., 2006, Cruz-Romero et al., 2007). Certain bacterial species are predominant in milk and dairy processes compared to others, they pose the ability to survive in hostile environment, form spores and biofilms, resist cleaning processes (Buehner et al., 2015). Such species need focus of dairy manufactures and researchers, where they need to investigate and explore the effect external interventions such as non-thermal techniques alone or in combination with thermal treatments to destroy these organisms. In the current study, we conducted experiments on the effectiveness of two cavitation techniques: ultrasonication and hydrodynamic cavitation, combined with heat, to inactivate the common dairy sporeformers and their endospores. Following are the main objectives of the study.

- To study the power ultrasound treatment and/ or hydrodynamic cavitation combined with pasteurization of milk inoculated with the thermally resistant

bacilli and their spores for studying their resistance to the combined treatments

- To trace the progression of the most resistant spore former during spray drying process using pilot scale milk spray dryer.

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CHAPTER 1

LITRETURE REVIEW

Dikshi Bawa

SPOREFORMERS AND SPORES IN DAIRY ENVIRONMENT

Milk is a source of nutrition for human beings, contains high water content along with neutral pH (Quigley et al., 2013), which makes it a good substrate for bacteria to grow. When milk is produced in udder it is clean and sterile (Tolle, 1980), however as it comes in contact with outer environment while milking, storage, transportation and processing, it starts getting contaminated. Common sources of contamination at farm can be soil, water, animal's outer body, milking equipment, milking practices, and time and temperature of storage. Microbiota of raw milk contains both pathogenic and spoilage bacteria. Milk drawn aseptically contains microbes at an average level of $<10^3$ CFU/mL, and as it gets exposed to contaminated environment, the bacterial load may go up as high as $>10^5$ CFU/mL, which may also be an indicator of poor sanitary conditions (Şenel and Gürsoy, 2014). Thermal processes such as pasteurization and higher heat treatment have been validated to kill pathogens of milk. However, heat resistant spore formers and their spores may survive these thermal treatments, adhere to stainless steel surfaces, and cause cross contamination of milk and subsequently dairy products leading to product quality and safety issues. Several endospore forming bacilli can survive heat treatments as well as other adverse process conditions (Postollec et al., 2012), and

can continue to grow in post heat treatment processes. They can be aerobic or anaerobic, and cause spoilage in dairy foods, even at low temperatures and water activity. There are 5 different classes of spore-forming bacteria which are *Bacilli, Clostridia, Erysipelotrichia, Negativicutes* and *Thermolithobacteria* (Galperin, 2013, Zhang and Lu, 2015). Out of these, *Bacilli* and *Clostridia* remain most consistent and dominant species that made them most important and relevant to dairy industry. *Bacillus, Geobacillus,* and *Clostridium* are gram-positive bacterial genera that have been extensively studied for their resistance against heating. More recent genera are *Paenibacillus, Brevibacillus* and *Anoxybacillus* that have shown the ability to form endospores. Some of these organisms are aerobic bacteria and some are strictly anaerobic. They can grow at various temperatures and some can survive extreme heat and extreme cold conditions.

Bacillus Genera

Bacillus is a genus, which has various heat resistant species that are gram positive, aerobic spore forming bacteria that can form spores under various unfavorable and stressful conditions. They can grow and survive in various natural conditions of air, water and soil, and also under various adverse conditions such as high and low temperatures, acidic and alkaline environments. Their spores are dormant in nature and pose ability to geminate when the environment becomes favorable. The bacteria have various species which are thermoduric in nature. Thermoduric bacteria can further be categorized as thermoduric mesophiles, thermophiles, and psychrotrophs depending upon their optimum growth

temperatures. Thermoduric mesophiles can grow at varying temperature range with 25 to 37°C as optimal range, however certain species can grow and survive under extreme conditions (Jay, 2012). Thermoduric thermophiles can survive pasteurization and can grow at around 55°C. Scott et al., (2007), reported the optimal growth temperature for thermophiles to be 40° to 65°C. Their survival is not only limited to extreme temperatures, but can also be extended to extreme pH that can be as low as pH 2 and as high as pH 10 (Drobniewski, 1993). Once they survive common heat treatments such as thermization and pasteurization, they get carried over to the final product and result in spoilage and other sensory defects. Some species are also capable to survive ultra-high temperature or ultra-pasteurization. Thermoduric psychrotrophs can grow and survive low temperatures and are capable to cause spoilage under refrigerated conditions. *Bacillus* is one of the most common genera present in refrigerated milk (Shehata et al., 1983).

Sporeforming bacterial group broadly includes several genera including *Bacillus, Brevibacillus, Paenibacillus, Geobacillus, Solibacillus, Ureibacillus, Virgibacillus, Lysinibacillus, Psychrobacillus* and *Anoxybacillus*. Some species such as *Bacillus, Geobacillus, Anoxybacillus* etc., are more significant to dairy industry. Bacilli can further be subcategorized into spoilage and pathogenic bacteria. *Bacillus cereus* and *B. anthracis* are classified as pathogenic species*,* whereas rest all species are non-pathogenic in nature. Common non-pathogenic species, which are found to be significant for dairy industry, are *B. subtilis, B. pumilis, B. stearothermophilus, B. coagulans, B. sporothermodurans* and other are *Brevibacillus bortelensis,*

Paenibacillus and *Anoxybacillus flavithermus*. Some of these organisms such as *Geobacillus stearothermophilus, B. licheniformis, A. flavithermus* etc. can grow between mesophilic temperatures to thermophilic range. Heat resistant spores of *G. stearothermophilus* and *A. flavithermus,* and their germinated cells are more common from powder plants (Scott et al., 2007a, Burgess et al., 2014, Buehner et al., 2015)

Various species of *Bacillus* are capable of sporulation. Sporulation is a mechanism of survival for the bacterial cells in response to adverse conditions due to stress and starvation. Spores form as an end product of sporulation process, which results in mother cell lysis to release spores (Setlow and Johnson, 2013). During this process, an asymmetric cell division occurs in which the mother cells engulfs the forespore. Multiple layers are then formed around the spore, between their inner membrane and outer membrane, which include a cell wall, a thick peptidoglycan cortex with a complex protein coating. The spore resistance and the DNA shielding of spores is due to acid soluble spore proteins, which are synthesized during sporulation process (Setlow and Johnson, 2013). The resistance is further linked to combined actions of multiple factors such as outer and inner spore coat, peptidoglycan cortex, spore proteins, and dipicolinic acid etc., (Burgess et al., 2010b). Studies conducted by Beaman and Gerhardt (1986) evaluated the factors affecting spore heat resistance. They found that thermal adaptation can impact spore resistance by reducing the water content and increasing wet density, and by

mineralization where calcium remineralized protoplasts were more dry, and hence, were more heat resistant (Beaman et al., 1982, Beaman and Gerhardt, 1986).

Once the conditions become favorable, spores start converting themselves to vegetative cells by activating themselves first, then germinating, and multiplying (Setlow, 2003).

Bacillus licheniformis

Bacillus licheniformis is one of the most common *Bacillus sp.* prevalent in the US dairy industry (Boor et al., 2002; Buehner et al.2014). The organism is a gram positive, facultative anaerobic, mesophilic rod shaped bacterium with an ability to produce spores resulting in food spoilage. *Bacillus licheniformis* has an optimum growth temperature of 30°C, but can grow at a lower temperature of 15°C, with a temperature as low as 6°C and the highest temperature of 55°C (Nissen et al., 2001, Burgess et al., 2010b). It was also identified among few species of *Bacillus* that have heat stable strains, which are capable of producing spoilage enzymes and toxins (De Jonghe et al., 2010). They were reported to have intermediate, but substantial proteolytic activity, and the ability to ferment lactose, utilize citrate starch and casein, reduce nitrate to nitrite, and showed strain-to-strain variation (Carlin, 2011). Species other than *Bacillus cereus*, including strains of *Bacillus licheniformis* are also found to produce toxins which make them more critical for human health (Pedersen et al., 2002).

Studies done by Buehner et al., (2015) found *B. licheniformis* as a predominant species in both summer and winter in mid-west dairy farms as well as

nonfat dry milk manufactured in mid-west dairy processing plants. These studies indicated that *B. licheniformis* could contaminate raw milk at farm level, which was further processed through powder manufacturing process to produce nonfat dry milk. Watterson et al., (2014) investigated the presence of thermophilic sporeformers in a northeastern powder manufacturing plants in which the collected samples of raw material, process, and final product were analyzed and showed the presence of mesophilic sporeformers including *B. licheniformis*. In a more recent study, VanderKelen et al., (2016) also found *B. licheniformis* as the most predominant species in milk and milk powder. Rückert et al., (2010) conducted a random amplified polymorphic DNA (RAPD) based survey on milk powder samples collected from 18 different countries found a strain of *B. licheniformis* as one of the two dominant stains. Another study conducted by Murphy et al. (1999), used a treatment of 77°C for 15s to kill thermophilic bacilli in milk, which was found inadequate to kill spore formers including *B. licheniformis*. They also found *B. licheniformis* in the final effects of evaporator with the temperature ranging between 49°C to 62°C. Heat resistance of spores of *B. licheniformis* was found related to protoplast dehydration, mineralization, and thermal adaptation (Cazemier et al., 2001). It was reported as the predominant species in ESL milk treated at 127°C for 5s by direct steam injection (Blake et al., 1995). Phillips and Griffiths, (1986) found *B. licheniformis* among raw milk isolates obtained at 55°C. Similarly, Crielly Williamson, (1995) also found *B. licheniformis* in raw milk. It was found in milk powder used in recombination process where it was linked to food poisoning

(Griffiths, 1995). Some toxin producing strains were also isolated from processed baby foods samples and raw milk samples (Salkinoja-Salonen et al., 1999).

Bacillus coagulans

Bacillus coagulans is a gram positive organism, which is one of the major *Bacillus* species responsible for spoilage in milk and dairy products (Robinson, 2005). The organism is a gram positive, facultatively anaerobe, motile rod shaped thermoduric bacterium with an ability grow under both mesophilic and thermiphilic conditions and to form spores and cause spoilage in milk and dairy products. *Bacillus coagulans* has an optimum growth temperature between 40 and 57°C, and optimum growth pH of 7.0 (Burgess et al., 2010b, Burgess et al., 2014). The maximum and minimum temperature for growth is between 57- 61°C and 15-25°C, respectivelly, and a pH range of pH 5.5 to 8.5.

Bacillus coagulans is capable to ferment lactose to lactic acid (Vecchi and Drago, 2006). High acid producing abilities of *B. coagulans* makes it an important organism in causing flat sour spoilage of canned products. Some stains of *B. coagulans* are found to cause coagulation and flat sour defect in concentrated milk by produing lactic acid without gas formation, which made this organism of econimic interst (Nakamura et al., 1988, Palop et al., 1999, Wang et al., 2009, Vercammen et al., 2012a). Spores of *B. coagulans* can be better protected in ionic solutes of NaCl and $CaCl_2$ at higher concentrations as compared to low water activity (Considine et al., 2008). They are more resistant in neutral foods as compared to acidic ones (Islam et al., 2006). In addition, due to its ability to form spores, some

stains of *B. coagulans* resist high temperature and human gastric environment. According to some recent studies, this has made these stains an area of interest for probiotic benefits delivering in human body (Majeed et al., 2016). *Bacillus coagulans* spores are heat resistant and are studied for their successful inactivation by introducing other interventions such as ultrasonication, ultraviolet irradiations, and high pressure processing (Wang et al., 2009, Gayán et al., 2013, Khanal et al., 2014a) .

THERMODURIC BACTERIA IN DAIRY PROCESS ENVIRONMENT

Raw milk

Milk is a source of nutrition and provides growth environment to various spoilage and pathogenic bacteria. In general, a high bacterial load >10^5 CFU/mL is an indicator of poor sanitary conditions. Spoilage organisms can be psychrophilic, mesophilic or thermophilic in nature and can show exponential growth during their log phase. Some of these bacteria are spore formers, which pose the ability to form spores when the environment becomes unfavorable. Raw milk microflora is very important as the milk further gets converted to various longer shelf life product such as cheese and milk powders. Common sources of contamination of milk is feed, soil, bedding, dung and feces, unclean milking utensils and poor milking practices (Chambers, 2002). The filter cloth of milking machines, green crop and fodder samples showed high concentrations of heat resistant spores with *B. licheniformis* as one of the predominant species (Scheldeman et al., 2005). Feed samples and milking equipment can be the main source of contamination and can behave as an entry point

for contamination in new stream of milk (Scheldeman et al., 2005). Buehner et al., (2014) found total bacterial count as high as 5.85 log and spore count as high as 6.33 log in corn silage. Spores can enter from dirty udders, manure, soil and even from unclean paper towels, and as few as a one spore is capable to cause contamination in milk (Martin, 2014). Soil residues on teats resulted in contamination of >Log 3 CFU/L of spores in milk (Vissers et al., 2007). Teat cleaning using moist and clean towels and wiping them with dry ones can significantly decrease spore load by 95% (Magnusson et al., 2006). Common nonpathogenic sporeforming species of bacteria associated with raw milk constitute *B. subtilis, B. licheniformis* and *Clostridium tyrobutyricum* etc. Raw milk is supposed to be cooled quickly and stored at 7°C or less to delay spoilage and multiplication of bacteria (USDHHS, 2013). Certain species of *Bacillus* at levels more than 10^7 CFU/mL are capable to produce flavor defects when milk when milk is stored at higher than 7°C. *Paenibacillus polymyxa* and species of *Bacillus* such as *B. subtilis, B. amyloliquefaciens* and *B. claussi* are psychotropic aerobic sporeformers capable of spoiling raw milk (Moatsou and Moschopoulou, 2014). *Bacillus licheniformis* and *B. coagulans* are common thermophiles found in raw milk (Seale et al., 2015).

Heating, evaporation, and drying process

Studies conducted on contamination and growth of sporeformers and their spores have indicated that the genera such as *Bacillus, Geobacillus* and *Anoxybacillus* are capable to sporulate and germinate in the preheaters, regeneration sections, and later stages of evaporators. Plate heat exchangers, pasteurizer,

preheaters, and evaporator are the main sites where these organisms can grow and multiply (Stadhouders et al., 1982, Murphy et al., 1999, Warnecke, 2001, Scott et al., 2007a). Thermoduric thermophiles can easily grow and get favorable temperature conditions in the range of 45 to 75°C, which are found in pasteurizers and evaporators. Facultative thermophiles such as *B. pumilus* and *B. coagulans* can easily grow in dairy processes at this temperature range, whereas *A. flavithermus* and *G. stearothermophilus* can grow in powder manufacturing lines (Burgess et al., 2014). This can result in fast multiplication and contamination of final product up to a level of 6 log CFU/mL. The contamination can go up when these end products enter the recirculation loop as rework or in the manufacturing of secondary food products. Milk powder is one of the main ingredient of dairy industry that is used in the manufacturing of dairy and food products. Since milk powder manufacturing includes pasteurization of milk followed by preheating, evaporation, and drying, so it becomes an area of concern for dairy manufacturers. During longer production runs, the spore count was found to go high during last few hours. Scott et al., (2007) found spore counts from <1 log CFU/mL to up to 4.1 log CFU/mL after 18 hours run. Similar to that Murphy et al., (1999) found significant increase in these organisms in evaporators after 8 hours run. They also found mid-section of evaporators showing *G. stearothermophilus,* and final effects of evaporators showing *B. licheniformis* growth. Watterson et al., (2014), however, found a limited evidence of spore count increase by the end of powder manufacturing run. One of the factors causing contamination can be physical concentration of the product by evaporation process.

In addition to that, recirculation of concentrate and additional heating by steam injection can also result in fouling in equipment. These foulants can also cause biofilm formation and high number of spores in end product.

SPOILAGE OF MILK AND DAIRY FOODS

In milk and secondary dairy products

As milk is a rich source nutrient, it certainly is a source of growth of various microorganism. Therefore, quality of milk is always linked to the bacterial load it carries. Spore forming bacilli and their spores survive pasteurization and result in cross contamination. *Bacillus licheniformis* was found as most predominant species followed by *B. subtilis* and *B. sonorensis* in milk from Midwest region (Buehner et al., 2014). They also found a seasonal variation with summer being worse than that of the winter season. In pasteurized milk, *Bacillus* species can grow and survive under cold conditions, and can produce heat stable extracellular enzymes to cause slight deviations in aroma or flavor to severe product spoilage issues (Şenel and Gürsoy, 2014, Vithanage et al., 2016). These bacilli are linked to production of undesirable compounds such as toxins and enzymes, which can degrade fat, protein and carbohydrates in food. Proteinase activity, by *B. subtilis,* resulted in formation of texture defects and bitter flavor in yogurt (Mistry, 2001). Proteinase activity is also responsible for breakdown of protein and resulting in sweet curdling defect. *Bacillus subtilis* was also related to ropiness defect in fresh milk (Heyndrickx and Scheldeman, 2002). Some strains of *Bacillus* can produce biogenic amines, which

are capable to produce toxins that are harmful for health (ZaMaN et al., 2010). Some other studies also found toxin producing strains of *B. licheniformis* in milk (Salkinoja-Salonen et al., 1999, (Nieminen et al., 2007). These strains were primarily linked to the cows having mastitis or had mastitis in the past. Strains of *B. licheniformis* were found to produce a slimy extracellular substance resulting in spoilage of pasteurized milk and cream, and strains of *B. coagulans* were found to produce lactic acid resulting in spoilage milk products (Gilmour and Rowe, 1990). Lecithinase producing species of *Bacillus* are responsible for bitty cream defect. Some species can produce bacterial polysaccharides in milk, which cause ropiness defect.

The spoilage, thus, caused by species of *Bacillus* genera is not limited to milk, it can also pass through the process barriers of heat treatments and can contaminate final product that are pasteurized, UHT or sterilized milk or any subsequent dairy products such as yogurt, cheese and milk powders. Flat sour is a common in UHT milk, which is linked to heat resistant spore of *B. licheniformis* and *G. stearothermophilus* (Boor and Murphy, 2002).

In concentrated and dried milks

Thermophilic bacilli are important factor affecting the quality of concentrated and dried milks. During concentration and drying process, about a 10-fold concentration of milk occurs, hence even if there is no growth in process, the count would go from <50 CFU/mL spores to 500 CFU/g spores in milk powder (Rückert et al., 2004). They are an area of concern, causing spoilage of milk powders, and the

subsequent products made from reconstituted milk (Kwee et al., 1986). Proteinases and lipases from various *Bacillus* species also survive heat treatments and result in causing end product spoilage issues related to degradation of milk protein and fat (Chen et al., 2003). In addition to enzyme production, these organisms were found to produce acid impacting organoleptic properties of milk powders (Gopal et al., 2015). Initially it was believed that these spoilage organisms and their enzymes get suppressed in milk powders due to its low moisture content, however later on, it was found that spoilage in milk powder is caused by enzymatic reactions in addition to chemical changes (Renner, 1988, Chen, 2000). Vegetative cells and spores of various *Bacillus* sp. such as *B. licheniformis, B. subtilis, B. pumilus,* and *A. flavithermus* and *Geobacillus* spp. cause shelf life problems in milk powder, UHT milk, and retorted products (Watterson et al., 2014). They also indicated thermophilic sporeformers to be the primary organisms (77.5%) causing contamination in powder manufacturing process. *Geobacillus stearothermophilus* was linked to spoilage of evaporated milk due to acid coagulation at a pH level of around 5.2. Flat sour defect was also detected in canned evaporated milk by *G. stearothermophilus* (Kakagianni et al., 2016). *Bacillus coagulans* and *G. stearothermophilus* may cause cheesy flavor and odor in condensed and evaporated milk (Kalogridou-Vassiliadou, 1990). *Bacillus subtilis* is linked to production of nonacid curd, browning and bitter taste (Robinson and Itsaranuwat, 2002).

Standards and Regulations

Lab pasteurization count is one way to see the presence of thermoduric bacteria in milk. A general rule here is that if this count goes more than 500 CFU/mL, then there can be a major problem with thermoduric bacteria in milk supply (White et al., 2001). Spore concentration of 10^5 spores/gm or more was considered as low valued downgraded product (Seale et al., 2008). In general, thermophilic sporeformers were limited to a maximum of 2,000 CFU/g, thermophilic bacteria to <10,000 CFU/g, and thermoresistant SP (100°C, 30 min) to <500 CFU/g (Wehr et al., 2004). Grade “A” pasteurized milk and milk products have bacterial limits not to exceed 20,000 per mL (USDHHS, 2013). This could be an indicator of total thermoduric load in milk.

Evaporated, condensed and powdered milk do not have any microbial specifications in FDA Standard of Identity 21. Whereas, the US Standards for Grades specifies a maximum standard plate count of 75,000 per gram for U.S. standard grade non-fat dry milk (spray process) and 10,000 per gram for U.S. extra grade non-fat dry milk (spray process). It also specifies the maximum standard plate count is 10,000 per gram for instant non-fat dry milk (Karaman and Alvarez, 2014). Customers of export markets have set strict tolerances (<500 to <1,000/g) for thermophilic and mesophilic spores in dairy powders as per the US Dairy Export Council, therefore, to meet that it becomes an important factor to understand their proliferation and survival within dairy powder manufacturing process in order to control sporeformer numbers (Watterson et al. 2014).

CONTROL OF THERMODURIC SPOREFORMERS AND SPORES

Thermal Processes

Milk pasteurization and ultra-high temperature (UHT) treatments are traditional and most common heating processes to reduce bacterial load in milk and dairy products. In dairy industry, thermization is one of the heat treatment processes, which is used to increase the storage time of raw milk and kill psychrotrophs present in milk. Temperature of thermization is 63 to 65°C for 15-20s. The most common heat treatment done to kill bacteria in milk is pasteurization, which can be either high temperature short time (HTST) at 72°C for 15s using plate heat exchangers, or low temperature long time (LTLT) at 63°C for 30 min. Most of the thermoduric organisms and their spores are capable to survive pasteurization and can enter downstream processes. Pasteurization process, hence, inactivates pathogens only, whereas sterilization with a different time-temperature combination is a process that has the ability to kill not only pathogenic organisms but also spoilage organisms and their spores (USDHHS, 2013). Ultra-pasteurization and UHT sterilization are the processes, which have also been tested to reduce thermodurics in milk. Ultra-pasteurization treatment ranges between 120 to 135°C for 1-4 s and UHT treatment is for 140 to 145°C for 1-8 s (Mehta, 1980, Boor and Murphy, 2002). These temperatures are effective in the reduction of bacteria to levels as high as 9 log CFU/mL, whereas product treated up to this temperature profile is sometimes not desired by the consumers as it results in cooked flavor and aroma (Chapman and

Boor, 2001). Therefore, in spite of their ability to kill thermoduric bacteria, high heat temperatures are not recommended to treat heat sensitive foods like milk and dairy products as heating may cause loss of vitamins, protein denaturation, development of undesirable color, flavor and aroma resulting in changes in sensory, nutritional and functional properties of final product. This has prompted food manufactures to identify alternate ways of manufacturing high quality, nutritious and safe foods. Alternate methods include studying the effectiveness of various non-thermal techniques alone or in combination with existing heat treatments. Techniques such as cavitation, pulse electric field, non-thermal plasma, high pressure processing etc. have been tested for their effectiveness in bacterial reduction in milk and other dairy products.

Non-Thermal Processes of interest

Ultrasound is defined as the sound waves of low frequency with a frequency range between 20 kHz to 100 kHz. These waves are undetectable by human ears and are capable to produce high power levels, hence known as high intensity ultrasound or power ultrasound (Villamiel et al., 2009). They are capable to achieve adequate microbial inactivation equivalent to thermal processes (Chouliara et al., 2010b). Ultrasonication process is based on the principle of cavitation. When sound waves pass through a liquid medium, they produce alternating high-pressure (compression) and low-pressure (rarefaction) cycles. During the rarefaction cycle, high-intensity ultrasonic waves create small vacuum bubbles or voids in the liquid. When the bubbles attain a volume at which they can no longer absorb energy, they collapse

violently during a compression cycle. The phenomenon of this violent implosion is known as ‘cavitation’ and when it is generated by sound waves it is known as acoustic cavitation. The temperature and pressure are assumed to rise inside the bubble up to 5500°C and 50 MPa respectively, which can effectively result in microbial inactivation (Raso et al., 1998, Villamiel et al., 2009). Ultrasonication alone does not show very high destructive effect, therefore it is combined with heat to show higher lethality. This process is known as thermosonication. Similarly, to increase the effect of ultrasonication, it is combined with pressure which is known as manosonication. Manothermosonication is another process which is a combination of ultrasonication with both heat and pressure (Demirdöven and Baysal, 2008, Evelyn and Silva, 2015). High intensity power ultrasound, when used in milk, successfully resulted in inactivation of *Bacillus* spores. Static trials using batch ultrasonication was found effective in reduction of these spores in non-fat milk. (Khanal et al., 2014a).

Controlled Hydrodynamic Cavitation is a mechanically induced cavitation process, which is an alternative to acoustic cavitation. There is a formation of bubble during rarefaction cycle and rapid collapse of vapor-filled cavity during compression cycle in a fluid due to localized high-and low-pressure regions induced by mechanical means (Milly et al., 2007). The mechanical rotor, contains radial holes in it, is used as an equipment to generate cavitation by spinning in a liquid chamber. The temperature and pressure increase in hydrodynamic cavitation is similar to that of acoustic cavitation (Gogate et al., 2006). Milly et al., (2007) innoculated skim

milk with *Clostridium sporogenes* putrefactive anaerobe 3679 spores and processed it at 3000 and 3600 rpm rotor speeds, resulted in temperature rise from 48.9° to 104.4° and 115.6°C with colony forming units (CFU) reductions of 0.69 and 2.84 log cycles, respectively. Hydrodynamic cavitation resulted in better efficiency as compared to acoustic cavitation, from the point of view of energy consumption (Capocellia et al., 2014).

Bactofugation is a process of removal of bacteria from milk using a centrifugal separation mechanism. The principle of operation for a bactofuge is separation by centrifugal force based upon variation in density. Spores are comparatively higher in density (1.2 to 1.3 gr/l) which makes them easily removable compared to bacterial cells, which have less density and are more difficult to remove (Stack and Sillen, 1998). Overall 99% of bacterial reduction can be achieved by this process. A high speed bactofuge is designed to remove bacteria from warm milk at a temperature of 60°C and at a centrifugal force of 980,000 kPa (Farkye, 2004). Stack and Sillen (1998) have discussed about the location of bactofuge in the processing line. The bactofuge can be fitted in raw milk line before cream separation step or in skim milk line after cream separation step. It can also be fitted in the final standardized milk line. The hermetically sealed bactofuge is another type of bactofuge, which is an airtight bactofuge. It is preferred to add this bactofuge to standardized milk line to reduce total solid loss. The process of bactofugation is very similar to clarification, while bactofugation is more efficient at removing bacteria, while milk clarification can only remove large size impurities. This makes it more

advantageous over clarifier. However along with bacteria, it also removes of milk solids and reduces overall total solid content by 2-3%. The sludge coming out of bactofuge contains both solids as well as bacteria. This results in additional treatment steps for manufacturers if they want to separate solids from the sludge.

High Pressure Processing (HPP) is also termed as Ultra High Pressure Processing and High Hydrostatic Pressure. High pressure is used in this method at different temperature profiles to achieve bacterial reduction. The operating pressure varies from 100 MPa to 1000 MPa and the temperature can range from below 0°C to above 100°C (Farkas and Hoover, 2008). The growth of microorganisms is retarded and protein synthesis is inhibited at 20-180 MPa. At a pressure of 180 MPa, the loss to cell membrane begins with the rate of increase in inactivation directly proportional to the rate of increase in pressure. At greater than 300 MPa, the protein denaturation starts and inactivation of cells takes place. The germination of spores was also observed between 50-300 MPa (Lado and Yousef, 2002). The combination of high pressure processing with high heat treatment resulted in germination and inactivation of bacterial spores in high acid food products (Vercammen et al., 2012a, Daryaei and Balasubramaniam, 2013b). A 7-8 log bacterial spores reduction was achieved when 700 MPa pressure was combined with 121°C temperature for 1 min (Scurrah et al., 2006b). Up to 5 log reduction in *B. subtilis* and 2 log reduction in *G. stearothermophilus* spores were achieved in a model buffer system, when a pressure of above 300 MPa and a temperature of above 145°C for ~0.24 s was applied

(Delgado et al., 2013). The effectiveness of HPP was improved when combined with heat treatment.

Filtration process using membranes is one of the most important technique currently used in dairy industry. Filtration technique using membrane of pore size between 0.1 to 10 µm is known as microfiltration (MF). It is a physical separation process in which pressure is used to separate particles of different sizes from the liquid medium. MF process is classified into two types based upon their operating procedure. One is Dead-end mode MF in which feed flows on to the membrane and retains particles over the membrane when the permeate flux decreases (Cheryan, 1998). The other type of procedure is a cross-flow mode in which liquid flows parallel to membrane surface and the separated particles get swiped with the retentate and the flow rate remains steady for a longer time (Ripperger and Altmann, 2002, Mukhopadhyay et al., 2011). The latter results in less deposits on the membrane surface and hence preferred more. MF does not cause damage to any other milk constituents, so it is considered as an effective way of bacterial separation (Villamiel et al., 2009). Tetra Pak manufactures a commercial microfiltration unit named as Tetra Alcross® Bactocatch which is coupled with a heat treatment unit. This can be integrated in milk processing line for the removal of bacteria and spores from milk. MF has two major disadvantages, one is that this process results in fouling and biofilm formation on membrane surfaces. The other disadvantage is that it cannot be used for whole milk as the particle size of bacteria (0.2-7 µm) and fat globules (0.2-6 µm) is similar, so both get separates out by the same membrane. A

complete removal of *Clostridium tyrobutyricum* spores in skim milk was achieved and a 5 log reduction in raw milk at 50°C was achieved using a ceramic membrane of 1.4 µm pore size (Kosilowski and Mistry, 1990, Awad et al., 2010).

Pulsed electric field (PEF) is another non-thermal technique based on the pulsing power delivered. A high voltage of electric pulses from 20 to 80 kV per cm is used on the product placed between a set of electrodes. This technique requires low energy to operate and the contact time for which the food product remains in the field is also in microseconds, therefore the process doesn't result significant temperature rise. The inactivation of microbes takes place by generation of pores in cell membrane, which is known as electroporation, resulting in cell death due to leakage of cellular content including ions, metabolite protein and DNA in the environment (Pothakamury et al., 1997, Toepfl et al., 2007). Both positive and negative charges start accumulating in the cell membrane causing transmembrane potential when the cell is exposed to electric field. When the field strength reaches above critical transmembrane potential, it results in electroporation (Evrendilek, 2014). The equipment used for PEF treatment includes a generator to produce high voltage pulses connected with electrodes placed in a treatment chamber. The electrodes are connected to each other using non-conductive material to prevent electric flow between them. PEF is effectively used in high acid products which have high electrical conductivity as well as high hydrogen ion concentration to transmit applied electric field strength. It is not found very effective for products containing air and fat, since both these components are poor conductors (Grahl and Märkl,

1996). The treatment of 40 kV per cm, 30 pulses and 2 μs pulse width using exponential decaying pulses resulted in increase in shelf life of raw milk (0.2% and 2% fat) by 2 weeks upon storage at low temperatures (Qin et al., 1998, Fernández-Molina et al., 2005).

Cavitation in dairy and food processing

The phenomenon of cavitation has been found applicable in various dairy food process operations such as microbial and enzyme inactivation, cleaning purposes, microbial disinfection, water treatment, filtration, homogenization, emulsification, crystallization etc., (Bermúdez-Aguirre et al., 2011). The use of cavitation to reduce thermally resistant organisms in dairy products is under research with many successful outcomes. Both ultrasonication and hydrodynamic cavitation have been used to reduce the population of spoilage bacteria. During early experiments, (Ordonez and Burgos, 1976) found the effect of ultrasound on reduction of *B. subtilis* spores. They found that ultrasonic treatment (20 kc, 1.2 A) for 10 min on 5 mL of medium was found effective to reduce D-value but had no effect on Z-value at 5.55°C. In more recent experiments, the combined effect of heat and ultrasonication was studied. Results showed a 1-2.1 log CFU/mL reduction in milk samples (Chouliara et al., 2010b). The process of adding pressure with ultrasonication process was found more effective in bacterial reduction (Piyasena et al., 2003). More recent studies found ultrasonication as an effective non-thermal technique to kill both thermoduric bacteria and their spores in milk (Khanal et al., 2014a, Khanal et al., 2014b). They also found an improved effectiveness when

ultrasonication was combined with heat treatment such as pasteurization. Unlike ultrasonication, hydrodynamic cavitation is studied for its effectiveness to reduce thermoduric organisms in milk to a limited extent. (Milly et al., 2007) evaluated the inactivation of *Clostridium sporogenes* spores in skim milk and found up to a 2.48 log CFU/mL reduction. They also found 3.1 log CFU/mL reduction when they treated *Bacillus coagulans* spores in tomato juice, and up to 5 log CFU/mL reduction when apple juice with *Lactobacillus plantarum* and *Lactobacillus sakei* cells, and *Zygosaccharomyces bailii* cells and ascospores, was treated at different process conditions of hydrodynamic cavitation. Another study was conducted on the effect of cavitation on reduction of pathogenic bacteria i.e. *E. coli* and *B. cereus,* as well as on yeast enzymes also showed its effectiveness to reduce these organisms (Save et al., 1997, Tsenter and Khandarkhayeva, 2012)

Ultrasonication VS Hydrodynamic cavitation

Ultrasonication is the process of cavitation using low intensity sound waves whereas hydrodynamic cavitation is generated by mechanical construction such as rotary impeller, venturi, orifice plate, or a throttling valve. This results in change in vapor pressure and generation of cavity in the liquid medium. Several researchers have tried to compare the effectiveness of ultrasonication to that of hydrodynamic cavitation. They have used Weissler reaction to assess the effectiveness of the two techniques. (Gogate and Pandit, 2001) used Weissler reaction to compare different cavitation equipment and found hydrodynamic cavitation more energy efficient than ultrasonication. They also found high cavitation yield and more versatility in

hydrodynamic cavitation than ultrasonication, for the machines they compared. (Capocellia et al., 2014) also found hydrodynamic cavitation resulted better efficiency as compared to acoustic cavitation, from the point of view of energy consumption. So hydrodynamic cavitation had better performance compared to acoustic cavitation equipment and was more efficient to scale-up for industrial applications. A hydrodynamic cavitation treatment of 30 min at 2 Lit/min resulted in 88% inactivation of microorganisms, whereas acoustic cavitation for 10 min at 0.17 Lit/min resulted in 95% microbial inactivation. The latter process required much higher power which made the former process more feasible for scale up (Crudo et al., 2014). Ultrasonication using small scale to large scale equipment showed limitations with process flow rates which made it unsuitable for handling large volumes and increased the interest in hydrodynamic cavitation. Hydrodynamic cavitator was found successful as an alternative for scale-up meeting all requirements established by ultrasonicator (Ashokkumar et al., 2011).

Other factors affecting effectiveness of non-thermal techniques

The effectiveness of non-thermal techniques also depends on the type of organism and the type of product to be treated. Bacterial cells have different shapes and sizes as well as different cell morphology, which impact their behavior against these treatments. Because of structural differences, spores were found to be more resistant then vegetative cells, gram positive bacteria showed more resistance than gram negative bacteria. Hence, the cell size and its shape impact the resistance levels with smaller cell being more resistant. Cocci were more resistant than rods with

some exceptions. Resistance of microbes to various process conditions also depends on their physiological state. The cells in stationary phase showed more resistance then the cells in exponentially growing or declining phase. Growth temperature and growth medium of the organism also found to have a significant effect on their resistance to non-thermal treatments (Evrendilek, 2014). The anti-microbial effect of these techniques was found to be influenced by the characteristics of food products as well. Characteristics such as pH, viscosity, electrical conductivity, water activity and overall composition of the food or environment of treatment medium influence the effectiveness of these processes (Oxen and Knorr, 1993, Patterson et al., 1995, Grahl and Märkl, 1996, Manas and Pagán, 2005, Evrendilek, 2014). Increase in cavitation effect due change in process parameters such as pressure increase can result in high microbial inactivation upto a certain extent, after that no further increase in inactivation was found. The authors hypothesized that this was due to the variation in ultrasonic fields, sensitivity of microorganism and product characteristics such as pH and total solids percentage (Piyasena et al., 2003).

CONCLUSION

Thermoduric spore formers and their spores show the ability to survive heat treatments and grow under adverse conditions. Presence of *Bacillus* spp. results in product defects such as sweet curdling, flat sour, ropiness, bitterness and fruity and unclean off-flavors. These organisms also cause process contamination by forming biofilms on equipment surfaces which are difficult to remove. Thermal treatments,

especially pasteurization and UHT are aimed to produce high quality milk and milk products by killing undesirable microflora. However, the effectiveness of these treatments is limited to certain species only. *Bacillus subtilis* and *Bacillus sporothermodurans* were found responsible for defects in UHT treated products. This limitation results in an immediate need of alternate methods to eliminate heat resistant organisms from dairy products and processes. Non-thermal technologies such as ultrasonication and hydrodynamic cavitation have recently gained more focus and attention due to their effectiveness against spore formers and spores as well as pathogens. These techniques when combined with thermal treatments show higher effectiveness as compared to when they are used alone. In the view of above discussions, non-thermal techniques show a great potential in inactivation of heat resistant spore formers and spores, and should be investigated more for their effectiveness in cell destruction and for the production of superior quality dairy products.

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CHAPTER 2

EFFECT OF ULTRASONICATION ON THE INACTIVATON OF THERMODURIC SPOREFORMERS IN SKIM MILK

DIKSHI BAWA

ABSTRACT

Thermally resistant sporeformers of *Bacillus* spp. can survive milk pasteurization process and cause spoilage problems in dairy foods. These sporeformers, upon entering the process are difficult to remove, and result in cross contamination of final products made out of that process. They can adhere to the walls of process equipment and form biofilms, which are more difficult to remove by regular cleaning techniques. Previous studies conducted in our lab established batch ultrasonication in combination with pasteurization as a technology to inactivate thermoduric vegetative cells of spore forming *Bacillus* spp (Khanal et al., 2014a; Khanal et al., 2014b)

The current study investigated the effectiveness of an ultrasonication process, in a continuous mode, to reduce thermoduric spore forming bacilli in skim milk. We hypothesized that ultrasonication can be used in a continuous mode, which would cause increase of cell membrane permeability leading to leakage of cell components thus causing cell death. Mid exponential phase vegetative cells of thermoduric *Bacillus coagulans* (ATCC® 12245) were inoculated in sterile skim milk at 5 log CFU/mL. Challenge studies were conducted to investigate thermal stability of these

cells against pasteurization. Inoculated skim milk sample was exposed to batch pasteurization (63°C for 30 min) and was validated for its resistance to pasteurization process. Inoculated skim milk was then passed through a continuous ultrasonicator with 22 mm sonotrode, 20 kHz frequency, and 1000 W power input (UIP1000hd, Hielscher USA, Inc.), at 86% (91.2 μm) amplitude and a flow rate of approximately 7.5 L/h and back pressure of 345 kPa resulting in an exposure time of 80 s per circulation or per pass.

Three different designs were conducted to investigate this process. The first experimental design included ultrasonication with continuous recirculation for up to 15 minutes with a cooling step at the exit of ultrasonicator flow cell. Samples were drawn after 6, 10, and 15 minutes. In the second experimental design, ultrasonication was applied under static conditions for 10 minutes. Design three included ultrasonication with discrete recirculation with 12 passes. Ultrasonicated samples were batch pasteurized to study the combined effect of ultrasonication and pasteurization. Brain Heart Infusion agar was used for plating the survivors. Experiments were conducted as replicates of two, and were repeated thrice. Statistical significance of the data was determined using SAS enterprise guide 9.2 software. A significant ($P < 0.05$) reduction was found in the log counts after treatments in design 2 and 3.

A 25% reduction of vegetative cells was found by lab pasteurization alone, whereas ultrasonication alone resulted in a higher inactivation of 92% in design three after 12 passes (80 sec exposure time per pass). Ultrasonication when combined with

pasteurization resulted in further increased inactivation to 99.98%. It can thus be concluded that a continuous ultrasonication process followed by pasteurization is effective to inactivate thermoduric vegetative cells of sporeformers such as *Bacillus coagulans*.

Key Words: Ultrasonication, *Bacillus coagulans,* skim milk, sporeformer

INTRODUCTION

Milk is one of the richest sources of nutrition and this makes it one of the most suitable foods for human consumption. At the same time, it also provides favorable conditions for food spoilage bacteria to grow and impact its quality. Due to this reason, safety and microbial quality of milk and milk products are important areas of concern. Among spore formers, *Bacillus* and its related species are some of the major Gram-positive aerobic microorganisms that are a concern for dairy industry. These bacilli are capable to grow at a wide range of temperature and can produce enzymes resulting in milk spoilage (Kalogridou-Vassiliadou, 1992). These spore formers do not get killed and get converted to spores under adverse conditions such as insufficient nutrition or inappropriate growth environment (Atrih and Foster, 2002). These spores when enter the process are difficult to remove and from there they can continue to enter products manufactured in that process. They are capable to produce extracellular enzymes which cause product spoilage (Seale et al., 2015). They can adhere to the walls of process equipment and form reoccurring biofilms which are more difficult to remove by traditional cleaning techniques (Flint et al., 1997, Chmielewski and Frank, 2003). These attachments of thermoduric bacteria on stainless steel can increase up to 100 fold due to milk fouling (Flint et al., 2001, Hinton et al., 2002).

Bacillus is one of the spoilage bacteria that has been studied for its resistance to heat treatment, formation of spores and germination process. Various species *B. licheniformis, B. coagulans, B. subtilis, B. pumilis, B. stearothermophilus* and *B.*

sporothermodurans are concern for dairy industry (Murphy et al., 1999, Rückert et al., 2004, Tabit and Buys, 2010, Karaman and Alvarez, 2014). More recently, species such as *Brevibacillus bortelensis, Paenibacillus, Anoxybacillus flavithermus*. and *Geobacillus stearothermophilus* have also gained attention (Scott et al., 2007a, Burgess et al., 2010b). *Bacillus coagulans* is one of the important strains of *Bacillus* spp. responsible for spoilage of dairy products (Robinson, 2005). The organism is thermoduric in nature and has shown the ability to form heat resistant spores. These spores are capable to grow between a temperature range of 15 – 60°C and a pH range from 5.5 – 8.5 (Burgess et al., 2010b). The major spoilage issues linked to this organism are due to its ability to ferment lactose to form lactic acid which results in spoilage due to high acidity (Vecchi and Drago, 2006, Vercammen et al., 2012b).

Thermal treatments such as pasteurization and UHT treatment, traditionally being used over decades, are capable to kill most of the spoilage and pathogenic bacteria, but they show a limited effectiveness against thermoduric spore formers and their spores (Faille et al., 2001, Ronimus et al., 2003, Rückert et al., 2004). This has prompted the need to identify alternate methods to reduce these organisms in milk. Alternate methods include studying the effectiveness of various non-thermal techniques and combining them with existing heat treatments. Ultrasonication, hydrodynamic cavitation, bactofugation, high pressure processing are some examples (Evrendilek, 2014).

Ultrasonication under static condition using acoustic cavitation, have been tested for its effectiveness in bacterial reduction in milk (Khanal et al., 2014b).

Ultrasonication uses sound waves which are categorized into high frequency ultrasound, which range from 2 MHz to 10 MHz which cause no destructive effect on foods, and low frequency sound waves ranging between 20 kHz to 100 kHz causing disruptive physical effects (Buehner, 2014). These low frequency waves are undetectable by human ears and are capable to produce high power levels ranging between 10 to 1000 W cm^{-2}, hence known as high intensity ultrasound or power ultrasound (Villamiel et al., 2009). They are capable to achieve adequate microbial inactivation equivalent to thermal processes (Chouliara et al., 2010b). Ultrasonication process is based on the principle of cavitation. When sound waves pass through a liquid medium, they produce alternating high-pressure (compression) and low-pressure (rarefaction) cycles. During the rarefaction cycle, high-intensity ultrasonic waves create small vacuum bubbles or voids in the liquid. When the bubbles attain a volume at which they can no longer absorb energy, they collapse violently during a compression cycle. The phenomenon of this violent implosion is known as 'cavitation' and when it is generated by sound waves it is known as acoustic cavitation (Leighton, 1995). The temperature and pressure are assumed to rise inside the bubble up to 5500°C and 50 MPa respectively, which can effectively result in microbial inactivation (Raso et al., 1998, Villamiel et al., 2009). Power ultrasound can be used in two different modes, other than sonication alone, to increase its destructive effect (Feng et al., 2008). These processes are, 1) thermosonication, when the inactivation is observed at lethal temperatures where heat and ultrasound are used in combination with each other, 2) manosonication

where sonication is combined with pressure to increase the lethal effect. When both heat and pressure are used in combination with ultrasonication, it is termed as Manothermosonication (Demirdöven and Baysal, 2008, Evelyn and Silva, 2015). The factors affecting effectiveness of these treatments are the equipment parameters (amplitude, inlet flow rate, temperature, pressure), exposure time, properties of medium, type of organism etc (Feng et al., 2011).

High intensity power ultrasound, when used in milk, successfully resulted in inactivation of *Bacillus* cells (Khanal et al., 2014a). Static ultrasonication alone resulted in up to 4 log reduction and when combined with pasteurization resulted in up to 5 log reduction (Khanal et al., 2014b). The current research investigates the effect of a continuous Ultrasonication process to reduce thermoduric sporeforming *Bacillus coagulans* (ATCC® 12245) in milk.

The objective of this research was to study the impact of power ultrasound in a continuous mode at different combination of amplitude and exposure time combined with pasteurization in inactivation of thermally resistance bacilli in skim milk, and to optimize the process to achieve maximum reduction. Ultrasonication can result in increase of cell membrane permeability leading to leakage of cell components thus causing cell death. A continuous ultrasonication equipment was used for the experiments. There are certain basic components used for the generation of ultrasound irrespective of the industry in which it is used. The equipment is an ultrasonic processor which is a complete ultrasonic system including a transducer and a generator (Hielscher, 2010). The generator fits on the top of the machine which

is a source of energy and is known as a power generator (Povey and Mason, 1998, Bermúdez-Aguirre et al., 2011). It produces electrical current which has a specific power rating measured in Watts. Power is a function of voltage (V) and current (I). Voltage is known as a potential energy of electrons and is measured in volts, and current is the net charge of electrons and is measured in amps (Hecht et al., 1997). The calculation of power is presented in Equation (2.1).

$$\text{Power} = \text{volts x amps} \tag{2.1}$$

An ultrasonic transducer is an electromechanical part of the equipment which transfers electrical power to mechanical oscillations. It is attached to a generator and is the central element where ultrasound is actually produced. It can transform 20 kHz of electrical energy, by vibrating at 20,000 mechanical cycles per s, into ultrasound energy of same frequency (Lee et al., 2003). The transducer used in the experiments was a piezoelectric transducer which is one of the most common types of transducer and is most efficient. It responds to electrical energy with better than 95% efficiency and is based on a crystalline ceramic material (Hielscher, 2010) (Bermúdez-Aguirre et al., 2011). The transducer is further connected to a booster which is a mechanical part mounted between transducer and emitter. It helps to control amplitude of the emitter by either increasing or reducing the mechanical amplitude. The last part of the equipment is known as emitter, which can be a bath or a sonotrode mounted to a horn, whose purpose is to emit the waves into the medium. The emitter used in the experiments was a sonotrode connected to a booster for transferring oscillations to the sample.

The functional principle of this equipment is to create a reversed piezoelectric effect by creating longitudinal mechanical oscillations with a frequency of 20 kHz by electric stimulation. The power input can be selected continuously upto 100% of the maximum power (1000 W). The sonotrode is placed inside the flow cell where it transfers increased oscillations (20,000/sec) into the continuously flowing liquid medium. These oscillations with 100% of mechanical amplitude (106µm) produces cavitation (Hielscher, 2010). This results in generation of high pressures and temperatures used in this process for microbial cell disruption.

MATERIAL METHODS

Culture Preparation and inoculation

Bacillus coagulans (ATCC® 12245) was obtained from American Type Culture Collection center (ATCC). The culture was revived in 5 mL Brain Heart Infusion (BHI; Oxoid) broth using the procedure recommended by ATCC. The cells were pelleted by centrifugation (Beckman Coulter Avanti J-E centrifuge) at 4500 x g for 30 minutes. The pellets were washed thrice by using phosphate buffer saline (PBS) at pH 7.4 and by centrifugation at 4500 x g for 15 minutes. Final washed suspension was stored in 1.8 mL cryo vials containing beads and glycerol at -80°C in deep freezer (NuAire ultralow freezer) for entire duration of experiments.

The organism was studied for its growth curve and generation time by using BHI Broth and their number was counted by plating done using BHI Agar. Mid exponential phase of vegetative cells of *Bacillus coagulans* (ATCC® 12245) was

determined from its growth curve. Optical density of the turbid broth was also measured by spectrophotometer (Thermo scientific Spectronuc 200) (Widdel, 2007). Cells were gram stained by using standard staining protocol and were viewed under microscope (Leica Microsystems) at 100 x magnification to verify for their purity and absence of spores.

A fresh culture was grown in BHI broth tubes for each experiment till its mid exponential phase. The cells were pelleted by centrifugation at 4500 x g for 30 minutes. Pellet was washed thrice by using phosphate buffer saline (PBS) at pH 7.4 and by centrifugation at 4500 x g for 15 minutes after each wash. Washed pallet was harvested by suspending it in 1 mL PBS and mixing on vortex mixer (Thermo scientific) for 30 s (Khanal et al., 2014b).

Skim milk preparation and inoculation

Two litres of reconstituted skim milk was prepared fresh for each experiment. Skim milk powder (Associated Milk Producers Inc.) was procured for making 11% solution. Reconstitution was done at 700 RPM at room temperature (~20 °C) using stirrer model IKA RW 16 (Fisher Scientific, NJ, USA). This milk was allowed equilibrate for some time and then autoclaved at 121°C for 15 min. The autoclaved milk was cooled till <10°C and was used for experiment. The freshly made cell suspension was inoculated at 5 log CFU/mL in this sterile skim milk sample.

Ultrasonication Treatment

A 1000 W UIP1000hd ultrasonicator equipment was procured from Hielscher USA, Inc. This device with BF2d22 (F) 22 mm sonotrode with frontal area 3.8 cm^2 in 164 ml volume flow cell, a B2-1.8 Booster with 106 µm (100%) amplitude, 20 kHz frequency and 1000 W Power input was used in this experiment (Figure 1). A positive displacement pump (Seepex pump) was used for input supply to ultrasonicator. Inoculated skim milk was transferred to Davis Dairy Plant for ultrasonication experiment. Ultrasonication treatment was conducted under three different processing designs.

Design 1 was made for ultrasonication with continuous recirculation with cooling. An ultrasonicator process in a continuous mode was designed (Figure 2). Inoculated milk was poured in tank 1 and pumped to ultrasonicator flow cell at a flow rate of 7.5 L/h. A back pressure of 345 kPa was maintained during recirculation. Milk was exposed to sonotrode at 86% amplitude which corresponds to 91.2 µm amplitude for 15 min. Samples were drawn after 6 min, 10 min. and 15 min. Exposure time was calculated by Equation 2.2 (Hielscher 2010).

$$\text{Exposure Time} = \frac{\text{Total run Time(min)} \times \text{ Volume of Flow cell (Lit)}}{\text{Flow rate } (^{\text{Lit}}/_{\text{min}})} \qquad (2.2)$$

Design 2 was made to conduct static experiments to validate the effectiveness of sonotrode used in ultrasonication by UIP1000hd. A 100 mL of milk sample was placed on ice water bath and was exposed to ultrasonication at 86% amplitude for 10 min.

Design 3 was made for ultrasonication with discrete recirculation without cooling. An ultasonicator process in a continuous mode was designed (Figure 3). Inoculated milk was poured in tank 1 and pumped to ultrasonicator flow cell at a flow rate of 7.5 L/h. A back pressure of 345 kPa was maintained during recirculation. Milk was exposed to sonotrode at 86% amplitude which corresponds to 91.2 μm amplitude for 80 s exposure time per pass. Milk flow from tank 1 to tank 2 is called as 1 pass (Figure 3). Flow rate and back pressure was adjusted to achieve desired exposure time. A total of 12 passes of treatment was provided to achieve 12 cavitation effects.

A validated cleaning in place (CIP) procedure was followed for effective cleaning and sanitation of the equipment. Before and after each experiment this CIP procedure was used for equipment cleaning (table 1).

Pasteurization

A control sample was drawn before the treatment and a ultrasonicated sample was drawn after the treatment. 3 mL of each of these samples was transferred to a sterile test tube with sterile threaded cap. Tubes were immersed in water bath preset at desired temperature for pasteurization. The time and temperature was monitored to achieve pasteurization treatment at 63°C for 30 minutes. The samples were then removed from water bath and kept in chilled water beaker till the temperature reached <5°C. These samples were used to calculate vegetative cells survivors after these treatments.

Enumeration of sporeformers

Both Control (before treatment) and treated milk samples were plated on BHI agar to calculate the survivors. Dilutions were made using 9 mL PBS solution. All counts were taken using standard pour plate technique (Laird et al., 2004). Plates were incubated inverted for 24 hours at 37°C. Colonies were counted as CFU/mL as per equation 2.3.

$$N = \frac{\Sigma C}{\{(n1 \times 1)+(n2 \times 2)\}d} \tag{2.3}$$

N = CFU/mL

ΣC = Sum of colonies on all counted plates

n1= Number of plates of lower dilution

n2= Number of plates of higher dilution

d= dilution level of n1 (lower count)

Percentage reduction of vegetative cell counts after each treatment was calculated as per equation 2.4.

$$\%\ \text{Inactivation} = \frac{\text{Counts before treatment} - \text{Counts after treatment}}{\text{Counts before Treatment}} \times 100 \tag{2.4}$$

Statistical Analysis

The experiments with three replicates were repeated twice. Statistical significance of the data at $P < 0.05$ was determined by ANOVA using the SAS 9.2.

MS excel was used to calculated mean, standard deviation and standard error, and to generate graphical representation of data.

RESULTS AND DISCUSSIONS

Bacillus coagulans (ATCC® 12245) culture preparation

Overnight grown culture of *Bacillus coagulans* (ATCC® 12245) up to its mid exponential phase was pelleted using BHI Broth and washed using PBS Solution. Washed pellet was suspended in 1 mL PBS solution and vortexed till mixed well. These harvested cells were used for inoculation up to 5 log CFU/mL in milk. The inoculated milk was studied for survival of this bacteria against pasteurization treatment before beginning ultrasonication treatment.

Pasteurization alone was not able to result in desired levels of reduction of these cells (Table 2). This was in line with previously done work by Khanal et al., 2014b in our lab. Similar observations have been made by other researchers which prompted a need to explore alternate methods to inactivate these organisms (Raso et al., 1994, Scurrah et al., 2006a, Wang et al., 2009, Vercammen et al., 2012b).

Ultrasonication Process Optimization

Bacillus coagulans (ATCC® 12245) inoculated in sterile skim milk and exposed to different process conditions to optimize ultrasonication. Preliminary experiments were conducted to achieve desired process conditions. Initial process design included ultrasonication with a continuous recirculation as described in Figure 2. Ultrasonication inlet flow was adjusted by adjusting inlet pump knob

between 0 and 1. Skim milk was dosed from the bottom of the flow cell and it came out from the top of the cell (Figures 1 and 2). A cooling water recirculation was provided to keep the flow cell cool. This setting provided a flow rate of 7.5 L/h. Ultrasonicator generator was adjusted to achieve an amplitude between 80% to 96%. As per manufactures recommendations, higher amplitude results in high wear and tear of sonotrode, so it was recommended to keep amplitude ≤ 96%. Back pressure of the equipment was adjusted to keep a constant pressure of 345 kPa by adjusting the back pressure valve manually. Temperature of inlet and outlet was monitored using type k digital thermometer. A digital LCD display Plug Power Energy Watt Voltage Amps Meter was used to measure input power. Exposure time was calculated from equation 2.2, which is the amount of time for which milk was in contact will ultrasound waves from inlet to outlet of ultrasonicator flow cell. Whereas, the total time was the time from inlet to outlet, which included inlet from balance tank, flowing inside pump, ultrasonication flow cell and through pipes. To increase the effectiveness of ultrasound, it was important to increase the exposure time and reduce the exposure volume, and to achieve optimal amplitude. Exposure time was increased by reducing the flow rate and flow volume. Amplitude was optimized by increasing generator energy and adding booster after transducer and before sonotrode.

In design one, a continuous recirculation ultrasonication treatment was carried out to establish correlation between process parameters. Inlet flow rate, amplitude and pressure were adjusted to achieve desired combination of parameters.

At a flow rate of 7.5 L/h and amplitude of 86% corresponding to ~900 W power, milk was recirculated for upto 15 min. Samples were drawn after 6 min, 10 min and 15 min. This was done to find out a base line for process conditions. The experiments were conducted with adding a chilled water bath after ultrasonication treatment, and cooling of milk before recirculating back to dosing tank was done. Design one provided preliminary information for smooth functioning of operating conditions. Due to low flow rates used and insufficient mixing in dosing tank, this design showed a limited effectiveness in reduction of bacterial cells.

Design two was made for conducting static trials (Figure 4). This was done to verify the effectiveness of sonotrode. As sonotrode is a wearable part of the equipment so there was a need to verify its effectiveness. The treatment was conducted for 10 min. at ~209 W resulted in complete inactivation of bacterial cells (Table 3).

Design three was the finally modified design used for ultrasonication experiments. An outlet tank was used for the collection of treated milk and milk passed from inlet tank to ultrasonicator flow cell to outlet tank was considered as one pass (Figures 3 and 5). Sterile tank was placed as tank 2 and was used for collection of milk after each pass. This milk was recirculated back to tank 1 at its low level. The process was repeated till 12 passes.

Ultrasonication Treatment Outcome

Ultrasonication after 12 passes (16 min) resulted in limited inactivation of thermoduric vegetative cells of *Bacillus coagulans.* A count of 4.04 ± 0.15 log

CFU/mL from an initial count of 5.20 ± 0.09 log CFU/mL was achieved by ultrasonication treatment with for 12 passes (Table 2). These results were equivalent to up to 92% reduction in *Bacillus coagulans* vegetative cells (Figure 6). This resulted in temperature rise from an initial level of 19.9ºC to 40.3ºC at 86% amplitude and ~850 W Power.

Ultrasonication followed by Lab Pasteurization Treatment Outcome

Pasteurization alone was insufficient to significantly reduce vegetative cells of *Bacillus coagulans.* A count of 3.88 ± 0.13 log CFU/mL from an initial count of 5.20 ± 0.09 log CFU/mL was achieved by lab pasteurization treatment at 63ºC for 30 min (Table 2). Ultrasonication when combined with pasteurization resulted in a count of 1.52 ± 0.16 log CFU/mL. The combined treatment resulted in a greater inactivation of over 3 log CFU/mL, in comparison to the reduction after pasteurization alone that was limited to 1.3 log CFU/mL (Table 2). Combination of Ultrasonication and pasteurization significantly reduced bacterial cells up to 99.98% (Figure 6).

These results were compared with previous research conducted by Khanal et al. 2014b. Static trials conducted using a 1000 W UIP1000hd ultrasonicator equipment by Hielscher USA, Inc showed similar results to that of the static trial conducted using a 5000 W, 20 kHz Vibra-Cell high intensity ultrasonic Processor VC 505 equipment by Sonics & Materials, Inc., CT, USA. Both the machines under their respective process conditions were found effective in reduction of vegetative cells of *Bacillus coagulans* by an average of ~5 log CFU/mL. The studies when

extended to trials in a continues mode showed where the reduction was up to 3 log CFU/mL. This showed that the equipment and the process were more effective under static mode then that of continuous mode.

CONCLUSIONS

Thermoduric strain of *Bacillus* species i.e. *Bacillus coagulans* was selected for studying the effectiveness of ultrasonication treatment. Continuous ultrasonication in combination with pasteurization was found as an effective method to reduce thermally resistant sporeformers of *Bacillus coagulans* over 3 log CFU/ml. The treatment was conducted for up to 12 passes which was equivalent to 12 cavitation effects. These experiments were conducted at pilot scale which increases the adaptability of this process at industrial scale. There can be scale up opportunities using various scale up equipment such as Hielscher ultrasound UIP 16000 with 16000 W of power supply and sonication units of 7 x UIP1000hdT. Scale-up can also be done using other cavitation techniques such as hydrodynamic cavitation which can further be explored for their application in dairy industry. The outcome of this study showed promising results for application of cavitation caused by ultrasonication in reduction of spoilage bacteria in milk.

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Table 1: CIP Protocol for UIP1000hd ultrasonicator equipment (SDSU Dairy Plant)

Step	Chemical	Quantity	Duration	Temperature
Rinse	Water	>10 L	10 min	100 F
Caustic Wash	Water Conquest	5 L 45 mL	20 min	110-120 F
Rinse	Water	>10 L	10 min	100 F
Sanitize	Water XY 12	5 L 12 mL	15 min	< 70 F
Production				
Rinse	Water	>10 L	10 min	100 F
Caustic Wash	Water Conquest	5 L 45 mL	30 min	110-120 F
Rinse	Water	>10 L	10 min	100 F
Acid Wash	Water Envirocid Plus	5 L 50 mL	30 min	110-120 F
Final Rinse	Water	>10 L	10 min	100 F

Table 2. Log CFU/mL of *Bacillus coagulans* cells before and after pasteurization, ultrasonication (after 12 passes) alone and in combination with pasteurization

Step	Mean*	SE**
Initial Count	5.20^{a}	0.09
Pasteurization[2]	3.88^{b}	0.13
Ultrasonication[1]	4.04^{b}	0.15
Ultrasonication[1] + Pasteurization[2]	1.52^{c}	0.16

* Mean of n=6
($^{a-c}$) Means without common superscripts are significantly different (P-value <0.05)
** SE= Standard Error
[1]Ultrasonication result after 12 pass
[2]Pasteurization at 63°C for 30 min

Table 3. Log CFU/mL of *Bacillus coagulans* cells before and after pasteurization, ultrasonication under static conditions

Step	Mean*	SE**
Initial Count	5.61[a]	0.02
Pasteurization[2]	4.76[b]	0.001
Ultrasonication[1]	0.00[c]	0
Ultrasonication[1] + Pasteurization[2]	0.00[c]	0

* Mean of n=6
([a-c]) Means without common superscripts are significantly different (P-value <0.05)
** SE= Standard Error
[1]Ultrasonication result after 12 pass
[2]Pasteurization at 63°C for 30 min

Figure 1. 1000W Ultrasonicator device with single sonotrode to be used in a continuous flow (Hielscher USA).

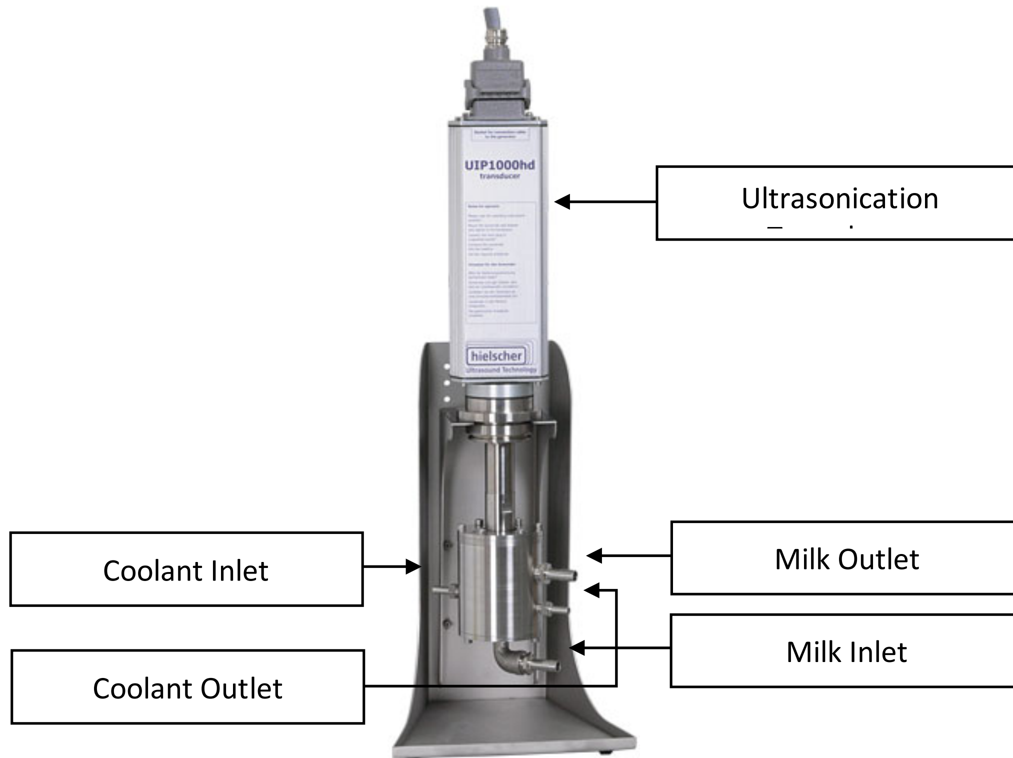


Figure 2. Process flow diagram of ultrasonication treatment for design 1 (preliminary trials) with ultrasonication of skim milk in a continuous recirculation mode.

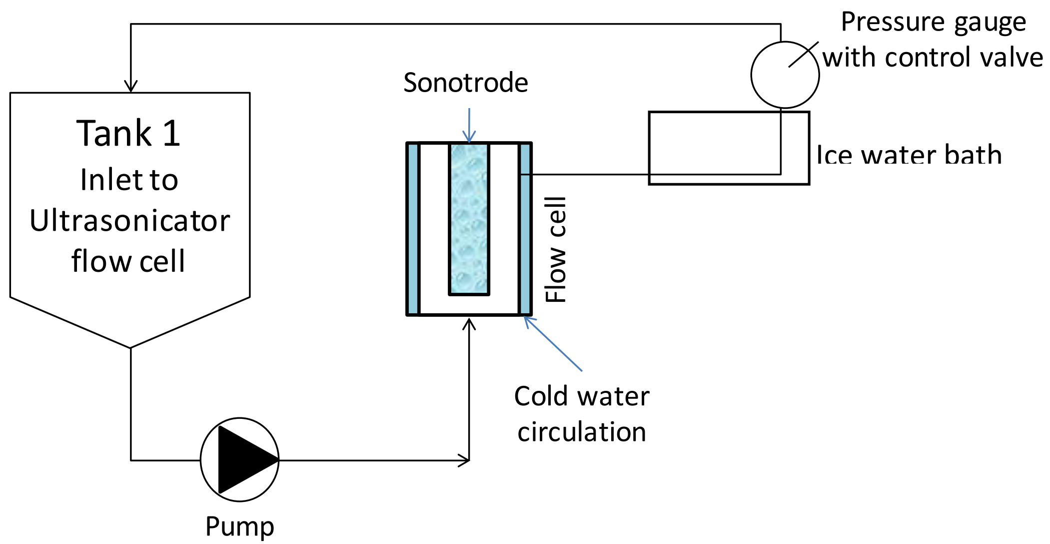


Figure 3. Process flow diagram of ultrasonication treatment for design 3 (continuous process trials) with ultrasonication of skim milk in a discrete recirculation mode.

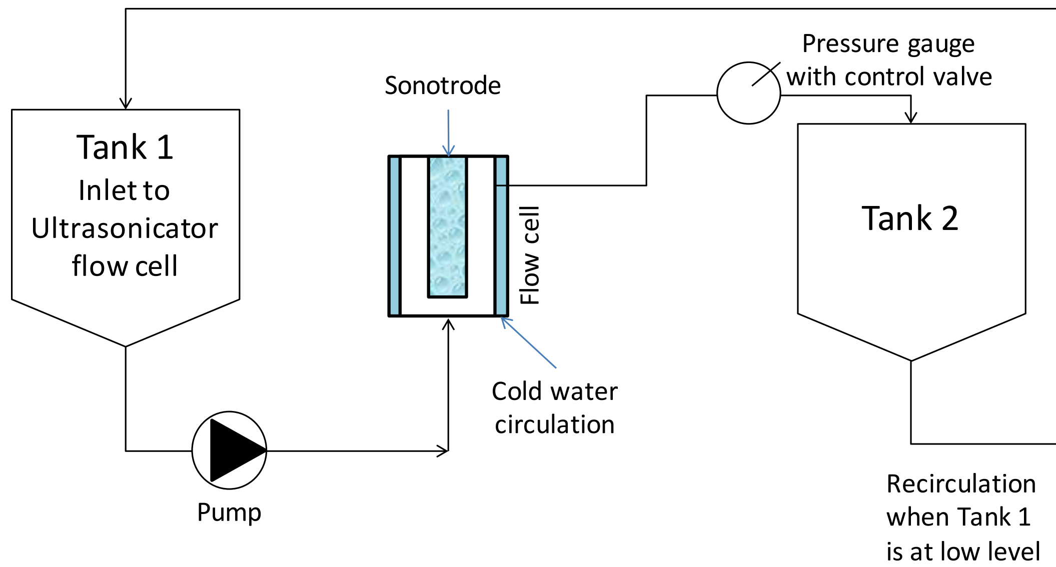


Figure 4. Static experiment design of ultrasonication of Skim milk using UIP1000hd, Hielscher USA, Inc.

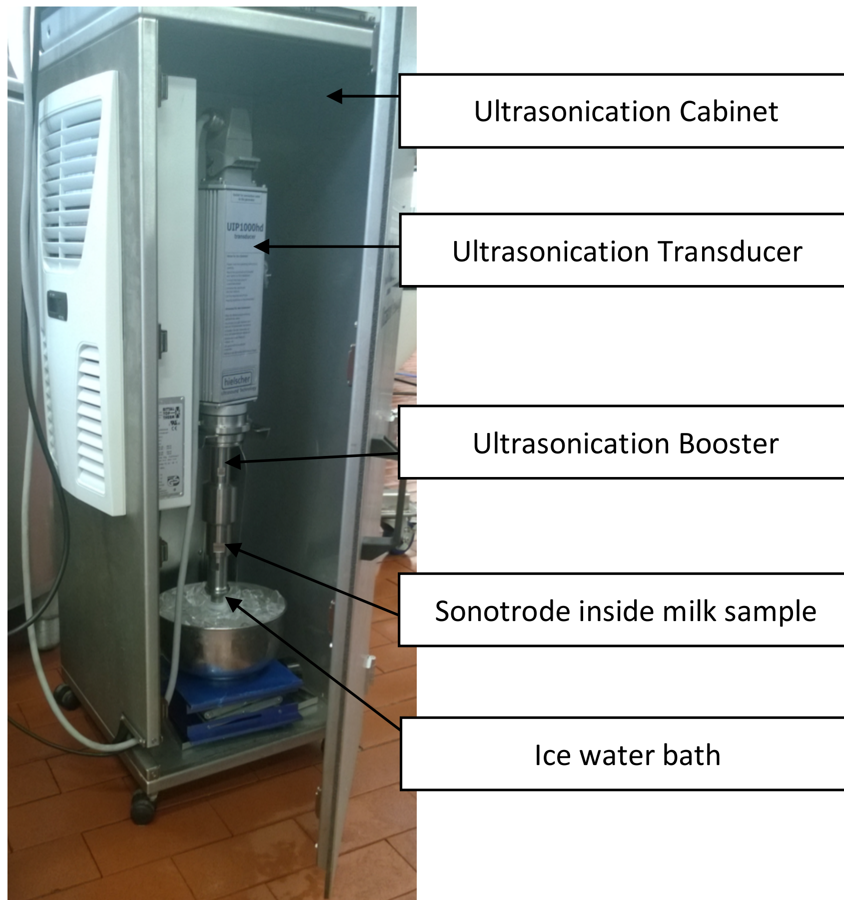
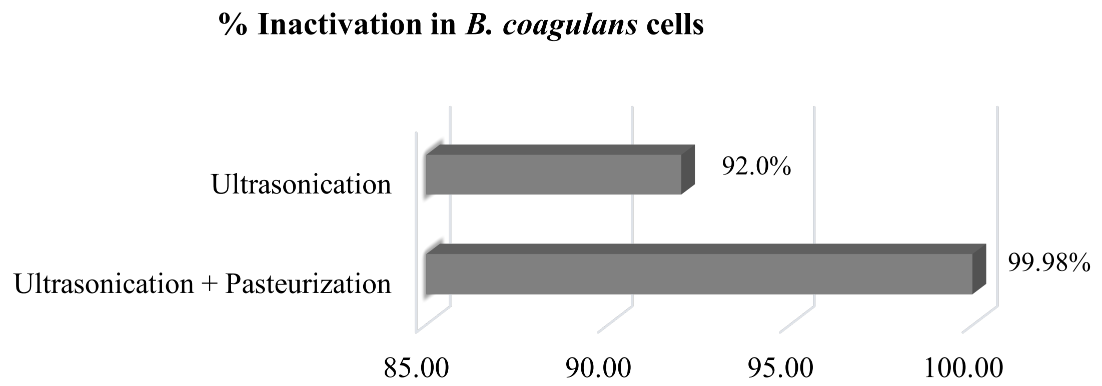


Figure 5. Ultrasonication treatment setup in R&D section of Davis Dairy Plant with inlet dosing tank, ultrasonication unit with an outlet line.

Figure 6. Levels of percent inactivation of *Bacillus coagulans* vegetative cells after ultrasonication treatment alone and in combination with pasteurization treatment.



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CHAPTER 3

EFFECT OF HYDRODYNAMIC CAVITATION ON THERMODURIC SPOREFORMERS IN SKIM MILK AND THEIR PROGRESSION DURING POWDER MANUFACTURING

DIKSHI BAWA

ABSTRACT

Thermoduric sporeformers show resistance to the commonly applied thermal treatments and later produce spores during further processing of milk. They survive pasteurization and can cause spoilage defects in dairy foods. The objective of current study was to investigate the application of controlled hydrodynamic cavitation in a continuous mode, and its combination with thermal treatments on the inactivation of thermoduric sporeformers in skim milk. Its impact on the final sporeformer counts of skim milk powder, in a simulated pilot scale skim milk manufacturing process, was also evaluated.

Mid exponential phase vegetative cells of *Bacillus coagulans* (ATCC® 12245) were inoculated in sterile skim milk at log 5 cfu per mL. Inoculated milk samples were passed at 60 Hz frequency and 200 L/h flow rate with 120 kPa back pressure, using APV Cavitator (supplied by SPX, Denmark) fitted with 4 row rotor in 6mm housing, resulting in exposure time of 22 sec per pass. The inoculated milk at 10°C was recirculated until 6 passes or 6 cavitation effects (a total exposure time of 132 sec) with an average inlet and outlet temperature rise up to 68°C and 82°C

respectively after the 6th pass. Samples were kept in an ice bath during the treatments for temperature control. For studying the combined effect, post cavitation samples were exposed to lab pasteurization (63°C for 30 min). Brain Heart Infusion Agar was used to plate the survivors. Experiments were conducted as replicates of two, and were repeated thrice. Statistical significance of the data was determined using SAS software. A significant reduction ($P < 0.05$) was observed in the bacterial counts after the treatments. The 6 pass cavitation effect alone, resulted in 0.97 log CFU/mL survivors. Additionally, the combined effect of cavitation and pasteurization further reduced the survivors to only 0.67 log CFU/mL. The results revealed that 6 pass cavitation effect alone or in combination with pasteurization was very effective in inactivating thermally resistant vegetative cells of *Bacillus coagulans* by 99.963% and 99.996% respectively.

Another set of experiments were repeated at low temperatures where passes were continued till the temperature of product at outlet increased to 72°C. Vegetative cells of *Bacillus coagulans* (ATCC® 12245) were grown up to their mid exponential phase and were inoculated in sterile skim milk at log 5.6 cfu per mL at 10°C. This milk was passed through APV Cavitator. Brain Heart Infusion Agar was used to plate the survivors using standard plating technique. Effect of cavitation on protein denaturation was also studied on these samples by measuring denatured protein percentage at pH 4.6 using Kjeldahl method. Experiments were conducted as replicates of two and were repeated thrice. Statistical significance of the data was determined using SAS software. A significant reduction ($P < 0.05$) was observed in

the bacterial counts after the treatments. After this treatment, a 1.70 log survivors were left. Denatured protein percentage was 75.75% before cavitation, which went up to 78.40% after cavitation. It can thus be concluded that continuous hydrodynamic cavitation effect, along with the rotor-liquid friction which generated controllable heating, was highly effective in inactivating thermally resistant vegetative cells of *Bacillus coagulans* by 99.989% with only 2.65% increase in denatured protein percentage.

Experiments were also conducted to study the progression of vegetative cells of *Bacillus coagulans* (ATCC® 12245) in cavitated milk up to the final spray dried skim milk powder manufacturing step. For this, *Bacillus coagulans* (ATCC® 12245) was grown up to its mid exponential phase, and were inoculated in sterile skim milk at log 2.7 CFU per mL. The spiked milk samples were passed through the APV Cavitator (SPX, Denmark) at pre-validated conditions. The spiked skim milk samples, without any cavitation treatment were considered as control. Both untreated and treated samples were concentrated to 30% total solids using a bench top rotary evaporator, and dried to 95.5% using Niro spray dryer. Brain Heart Infusion agar was used to plate the survivors using standard plating techniques. Effect of cavitation on protein denaturation in milk was also looked at by measuring denatured protein percentage at pH 4.6 using Kjeldahl method. Statistical significance of the data was determined using SAS software. A significant reduction ($P < 0.05$) was observed in the bacterial counts after the treatment. After 6 cavitation effects, only about 0.95 log survivors were left in milk, which finally resulted in 2.5 log CFU/g in the skim milk

powder. In comparison, the bacterial counts were significantly higher at 5.0 log CFU/g in skim milk powder made from control (non-cavitated) samples. Denatured protein percentage went up by ~8% after cavitation. The current study, though a simulation, helped to demonstrate the usefulness of including a hydrodynamic cavitation step in reducing the overall counts in skim milk powder by over 2 logs.

Last set of experiments were conducted using skim milk and skim milk concentrate samples collected from a concentrate milk manufacturing company. The samples were already pasteurized at their end and contained a mixed population of thermoduric sporeformers and spores. A cavitation treatment up to 6 passes was conducted and the effect on these samples was evaluated. Brain Heart Infusion agar was used to plate the survivors using standard plating techniques. Effect of cavitation on protein denaturation in milk was also studied by measuring denatured protein percentage at pH 4.6 using Kjeldahl method. Statistical significance of the data was determined using SAS software. A significant reduction ($P < 0.05$) was observed in the total bacterial counts after the treatment, however no significant reduction was observed in total spore counts. Higher increase in denatured protein was observed in concentrate samples compared to skim milk samples. This study helped to demonstrate that cavitation at set conditions was more effective on skim milk than skim milk concentrate, and 6 pass effect was found more effective on sporeformers than spores.

Key words: Hydrodynamic Cavitation, thermoduric sporeformes, skim milk

INTRODUCTION

The United States is one of the largest producers of milk in the world. As milk is a perishable commodity, it becomes very important to prevent its spoilage, which makes spoilage bacteria important for dairy industry. To increase the shelf of milk, it is processed and converted to various dairy ingredients and finished products. It is very important to reduce spoilage bacteria in the end products to improve the microbial quality, but also to prevent further spoilage of secondary products to which they are added (Gopal et al., 2015). *Bacillus* spp. represent aerobic sporeforming organisms that have been of concern for dairy food manufactures. Various *Bacillus* species are thermoduric in nature and survive pasteurization. These organisms are capable to form endospores that germinate and grow in post pasteurization process steps. Once they enter dairy foods, they cause spoilage during storage (De Jonghe et al., 2010, Khanal et al., 2014b, Moatsou and Moschopoulou, 2014). Their resistance to various physical and biochemical treatments, dormancy, ability to adhere to the surfaces of process equipment resulting in biofilm formation, and prompt germination under favorable conditions, make them important contaminants in dairy processes (Khanal et al., 2014a, Khanal et al., 2014b). During the manufacture of skim milk powder, as the milk gets concentrated, it results in concentration of these thermoduric organisms, as well (Rückert et al., 2004).

Bacillus species including *B. licheniformis, B. coagulans, B. subtilis, B. pumilis, B. stearothermophilus* and *B. sporothermodurans* are concern for dairy industry (Murphy et al., 1999, Rückert et al., 2004, Tabit and Buys, 2010, Karaman

and Alvarez, 2014). *Bacillus coagulans* is gram positive thermophilic nonpathogenic, but sporeforming bacteria, responsible for spoilage of dairy products (Robinson, 2005). The organism is thermoduric in nature and has shown the ability to form heat resistant spores. These spores are capable to grow between a temperature range of 15 – 60°C and a pH range from 5.5 – 8.5 (Burgess et al., 2010b). The major spoilage issues linked to this organism are due to its ability to ferment lactose to form lactic acid, which results in spoilage due to high acidity resulting in fermentation, off- flavor and aroma, and cloudiness in some foods (Vecchi and Drago, 2006, Doyle and Buchanan, 2012, Vercammen et al., 2012b).

Thermal treatment such as pasteurization and UHT treatment traditionally being used over decades are capable to kill most of the spoilage and pathogenic bacteria, but they show a limited effectiveness against thermoduric spore formers and their spores (Faille et al., 2001, Ronimus et al., 2003, Rückert et al., 2004). This has prompted the need to identify alternate methods to reduce these organisms in milk that include studying the effectiveness of various non-thermal techniques and combining them with existing heat treatments (Manas and Pagán, 2005, Evrendilek, 2014).

Cavitation is classified into four different types depending upon its generation mode. These different cavitation types are optic, particle, acoustic, and hydrodynamic (Gogate and Kabadi, 2009). Optic and particle are single bubble cavitation and have not shown any success in inducing any change in bulk liquids. Acoustic and hydrodynamic are the ones shown better results with multi-bubble

cavitation in producing desirable changes in processing environment (Mason and Lorimer, 2002, Gogate and Kabadi, 2009). These techniques offer immense potential to improve processing in an energy efficient way. (Suslick, 1989) defined cavitation as a mechanism of generation of cavities, their growth, and collapse resulting in high localized energy densities. This further results in pockets of energy with high temperature and pressure, but overall a controlled environment equivalent to ambient conditions (APV Cavitator Manual 2013).

Ultrasonication is one of the non-thermal technique based on acoustic cavitation, which is being tested for its effectiveness to kill sporeformers in milk. Static trials conducted by Khanal et al., (2014b) were found effective. A continuous ultrasonication was tested by us in a previous experiment of this study, and found effective in reduction of *B. coagulans* cells by up to 3 log CFU/mL (Chapter 2). However, ultrasonication, in general, showed some limitations in scale up, especially at higher flow rates. In trials conducted by Ashokkumar et al., (2011), a 4000 W 20 Hz Hielscher Systems GmbH UPI-4000 was used to study the effectiveness of ultrasonication on microbial cell disruption. They found ultrasonication as an effective technique in killing microorganisms however, reported a limitation in terms of processing volume. They could validate the equipment at low flow rates, which was, however, not comparable to process environment where tons of liters of milk was handled per day. This limitation triggered a need to explore hydrodynamic cavitation as an alternative to process large volume of milk (Ashokkumar et al., 2011). In another set of experiments, (Crudo et al., 2014) also used a small scale

sonochemical reactor to process 70 mL of liquid in a continuous mode. In some comparison studies conducted by (Save et al., 1997), ultrasonication was also found as a less energy efficient technique compared to hydrodynamic cavitation. They also observed that sonication generated higher heat compared to hydrodynamic cavitation, and the cell debris wherever needed to be removed were harder to remove in ultrasonicated samples compared to that of hydrodynamic cavitation treated samples. (Gogate and Pandit, 2001). They also found hydrodynamic cavitation more energy efficient than ultrasonication. (Capocellia et al., 2014) reported similar observations for hydrodynamic cavitation that resulted in better efficiency as compared to acoustic cavitation, from the point of view of energy consumption.

Controlled Hydrodynamic Cavitation is a mechanically induced cavitation process, which is an alternative to acoustic cavitation. It involves formation of bubbles during rarefaction cycle and rapid collapse of vapor-filled cavity during compression cycle in a fluid due to localized high-and low-pressure regions induced by mechanical means (Milly et al., 2007). The mechanical rotor contains radial holes in it, and is used to generate cavitation by spinning in a liquid chamber. The temperature and pressure increase in hydrodynamic cavitation is similar to that of acoustic cavitation (Gogate et al., 2006, Gogate, 2011). When the bubbles attain a volume at which they can no longer absorb energy, they collapse violently during a compression cycle. The temperature and pressure are assumed to rise inside the bubble up to 5500°C and 50 MPa respectively, which can effectively result in microbial inactivation (Suslick, 1989, Raso et al., 1998, Villamiel et al., 2009,

Khanal et al., 2014b). Thus, cavitation results in generation of high energy pockets along with shock waves and shear forces. The factors affecting bubble formation and bubble behavior in case of hydrodynamic cavitation could be the geometry of the equipment as well as the operating condition (Gogate and Pandit, 2005). The liquid is passed through a mechanical device with a certain type of construction such as throttling valve, orifice plate, venturi or a rotating impeller with holes (Figure 1). This causes the pressure to fall below the vapor pressure of the medium due to velocity variations at a certain operating parameters resulting in formation of cavities. Milly et al., (2007) innoculated skim milk with *Clostridium sporogenes* putrefactive anaerobe 3679 spores, and processed it at 3000 and 3600 rpm rotor speeds. This resulted in temperature rise from 48.9° to 104.4° and 115.6°C with colony forming units (CFU) reductions of 0.69 and 2.84 log cycles, respectively. In another research, hydrodynamic cavitation was found effective in disinfecting *E. coli*, *B. cereus* cells and spores in water on a lab scale rotary cavitation equipment (Tsenter and Khandarkhayeva, 2012). Crudo et al., (2014), reported up to 88% reduction in total bacterial count using hydrodynamic cavitation.

The main objective of our study was to investigate the application of controlled hydrodynamic cavitation in a continuous mode, and its combination with thermal treatments on the inactivation of thermoduric sporeformers in skim milk. Another objective was to include a hydrodynamic cavitation step, prior to skim milk pasteurization, and evaluate its impact on the final sporeformer counts of skim milk powder manufactured using cavitated skim milk, in a simulated pilot scale skim milk

manufacturing process. We hypothesized that the high-speed high shear effect of cavitation shock waves and generation of heat with pressure would cause rupturing of the bacterial cell wall resulting in greater cell death by combining with a thermal treatment. This would lead to elimination of majority of sporeformers at the skim milk initial handling stage itself, resulting in a low bacterial count skim milk powder. The APV cavitator manufactured by SPX was used to conduct the experiments. The equipment consists of a rotary impeller connected to a motor. The impeller is the part of cavitator responsible for cavitation. There is a rotor inside the housing, which contains radial holes. Milk flows through each of these holes, and upon its rotation results in cavitation (APV Cavitator Manual, 2013). The number of holes present in the rotor are directly proportional to cavitation produced. The equipment consists of three different types of rotor i.e. rotor with 2, 3 and 4 rows of holes. A Rotor with more holes provides larger cavitation volume, however, the shear force is also higher with more force, which may not be desirable for certain liquids.

MATERIAL METHODS

Culture Preparation and inoculation

Bacillus coagulans (ATCC® 12245) was obtained from American Type Culture Collection center (ATCC). The culture was revived in 5 mL Brain Heart Infusion (BHI; Oxoid) broth using the procedure recommended by ATCC. The cells were pelleted by centrifugation (Beckman Coulter Avanti J-E centrifuge) at 4500 x g for 30 minutes. The pellets were washed thrice by using phosphate buffer saline

(PBS) at pH 7.4 and by centrifugation at 4500 x g for 15 minutes. Final washed suspension was stored in 1.8 mL cryo vials containing beads and glycerol at -80°C in deep freezer (NuAire ultralow freezer) for the experiments.

The organism was studied for its growth curve and generation time by using BHI Broth and their number was counted by plating done using BHI Agar. Mid exponential phase of vegetative cells of *Bacillus coagulans* (ATCC® 12245) was determined from its growth curve. Optical density of the turbid broth was also measured by spectrophotometer (Thermo scientific Spectronuc 200) (Widdel, 2007). Cells were gram stained by using standard staining protocol and were viewed under microscope (Leica Microsystems) at 100 x magnification to verify for their purity and absence of spores.

A fresh culture was grown in BHI broth tubes for each experiment till its mid exponential phase. The cells were pelleted by centrifugation at 4500 x g for 30 minutes. Pellet was washed thrice by using phosphate buffer saline (PBS) at pH 7.4 and by centrifugation at 4500 x g for 15 minutes after each wash. Washed pallet was harvested by suspending it in 1 mL PBS and mixing using a vortex mixer (Thermo scientific) for 30 s (Khanal et al., 2014b).

Hydrodynamic Cavitation Treatment

An APV Cavitator (SPX Flow technology, Goldsboro, North Carolina) with 2, 3 and 4 row rotor with 3mm and 6mm hosing option was provided for conducting experiments. The equipment was capable to run at a frequency of up to 60 Hz and a flow rate of up to 1000 L/h with a back pressure of up to 10 bars. Temperature and

pressure gauges were provided at both inlet and out let of the equipment to monitor inlet and outlet temperature and pressure, respectively. A flow meter was provided to monitor flow rate and a back pressure value was provided to control back pressure. The equipment was connected to an inlet centrifugal pump to adjust incoming flow to cavitator.

A 10 L sample of sterile skim milk was inoculated with vegetative cells of *Bacillus coagulans*. Milk was then poured in a sanitized stainless steel tank for cavitation treatment. Design 1 was made for hydrodynamic with continuous recirculation. A continuous mode was designed and connected to an inlet pump and feed tank. Inoculated milk was poured in tank 1 and pumped to cavitator at a flow rate of 200 L/h. A back pressure of 120 kPa was maintained during recirculation. Skim milk was passed through APV Cavitator at 60 Hz frequency using 4 row rotor in 6mm housing (Figure 2). The milk containing bacteria was recirculated for 18 min. The experiments with three replicates were repeated twice. Exposure time was calculated by Equation 3.2 (Hielscher 2010), which was found equivalent to 132 s.

$$\text{Exposure Time} = \frac{\text{Total run Time(min)} \times \text{Volume of Flow cell (Lit)}}{\text{Flow rate } \left(\text{Lit}/_{\text{min}}\right)} \qquad (3.2)$$

Design 2 was made for hydrodynamic cavitation in a continuous mode with discrete recirculation (Figure 3). Inoculated milk was poured in dosing tank and pumped to cavitator at same process parameters mentioned above. Milk flow from dosing tank to outlet tank was considered as 1 pass and was treated for 6 passes equivalent to an exposure time of 22 s per pass.

Design 3, similar to that of design 2, was made for hydrodynamic cavitation in a continuous mode with discrete recirculation. Unlike design 2, number of passes were continued till the temperature reached 72ºC. All parameters were kept same as all above.

A validated cleaning in place (CIP) procedure was followed for effective cleaning and sanitation of the equipment. Before and after each experiment this CIP procedure was followed similar to that of ultrasonication experiments for cleaning of cavitator unit.

Pasteurization

A control sample was drawn before the treatment and a cavitated sample was drawn after the treatment. 3 mL of each of these samples was transferred to a sterile test tube with sterile threaded cap. Tubes were immersed in water bath preset at desired temperature for pasteurization. The time and temperature was monitored to achieve pasteurization treatment at 63°C for 30 minutes. The samples were then removed from water bath and kept in chilled water beaker till the temperature reached <5°C. These samples were used to calculate vegetative cells survivors after these treatments.

Milk Powder Manufacturing

For this, *Bacillus coagulans* (ATCC® 12245) were grown up to their mid exponential phase, and were inoculated in sterile skim milk at log 2.7 CFU per mL. The spiked milk samples were passed through the APV Cavitator at pre-validated

conditions for 6 passes at a flow rate of 200 L/h, a back pressure of 120 kPa at 60 Hz frequency using 4 row rotor in 6mm housing. The spiked skim milk samples, without any cavitation treatment were considered as control. Both control and treated samples were concentrated to 30% total solids using a bench top rotary evaporator, and dried to 95.5% using Niro spray dryer. Brain Heart Infusion agar was used to plate the survivors using standard plating techniques.

Enumeration of sporeformers

Both Control (before treatment) and treated milk samples were plated on BHI agar to calculate the survivors. Dilutions were made using 9 mL PBS solution. All counts were taken using standard pour plate technique (Laird et al., 2004). Plates were incubated inverted for 24 hours at 37°C. Colonies were counted as CFU/mL as per equation 3.3.

$$N = \frac{\Sigma C}{\{(n1 \times 1) + (n2 \times 2)\}d} \tag{3.3}$$

N = CFU/mL

ΣC = Sum of colonies on all counted plates

n1= Number of plates of lower dilution

n2= Number of plates of higher dilution

d= dilution level of n1 (lower count)

Percentage reduction of vegetative cell counts after each treatment was calculated as per equation 2.4.

$$\%\ \text{Inactivation} = \frac{\text{Counts before treatment} - \text{Counts after treatment}}{\text{Counts before Treatment}} \times 100 \tag{3.4}$$

Denaturation of protein

Treatment of skim milk or skim milk concentrate using hydrodynamic cavitation may result in denaturation of serum proteins due to generation of heat and high shear effect of cavitation (Walstra, 2013). The extent of denaturation is directly proportional to the amount of heat used (Fox et al., 2015). Effect of cavitation and resulting heat generation on protein denaturation in milk was also studied by measuring denatured protein percentage at pH 4.6. Two samples were analyzed in duplicates using Kjeldahl method and calculated using Equation 3.5.

$$\frac{\text{Total Nitrogen} - \text{Non Casein Nitrogen}}{\text{Total Nitrogen}}\ \% \qquad (3.5)$$

Statistical Analysis

The experiments with three replicates were repeated twice. Statistical significance of the data at $P < 0.05$ was determined by ANOVA using the SAS 9.2. MS excel was used to calculated mean, standard deviation and standard error, and to generate graphical representation of data.

Industrial samples related studies

After the completion of pilot scale cavitation experiments, trials were extended to industrial samples for studying the effectiveness of cavitation on mixed population of sporeformers and spores. Both skim milk and skim milk concentrate samples were collected for the manufacturing plants. Trials were conducted as per the conditions validated for ATCC Culture of *B. coagulans*, as described above

(Chapter 3, Page 11-12). Preliminary analysis was done to standardize the plating procedure for determining the bacterial counts in samples. MALDI-TOF was done to identify species. To evaluate the effect of hydrodynamic cavitation, 10 L samples were exposed to cavitation process at low temperatures, with the starting temperature of control at 8ºC and ending temperature of 72ºC. Temperature was monitored after each pass. A control and a final pass cavitated samples were analyzed for microbes and denatured proteins using standard protocols. The rise in temperature, during each pass through the cavitator, was also recorded. Concentrated samples were also dried after cavitation treatment to study the progression up to manufacturing of skim milk powder.

RESULTS AND DISCUSSIONS

Bacillus coagulans (ATCC® 12245) culture preparation

Overnight grown culture of *Bacillus coagulans* (ATCC® 12245) up till its mid exponential phase was pelleted using BHI Broth and washed using PBS Solution. Washed pellet was suspended in 1 mL PBS solution and vortexed till mixed well. These harvested cells were used for inoculation up to 5 log CFU/mL in milk. The inoculated milk was studied for survival of this bacteria against pasteurization treatment before beginning ultrasonication treatment.

Pasteurization alone was not able to result in effective reduction of these cells with 2.73 ± 0.20 log CFU/mL survivors from an initial count of 4.05 ±0.21 log CFU/mL (Table 1). Other researchers also found pasteurization alone was found as

insufficient technique to kill spoilage bacteria which led to a need to explore alternate methods in inactivation of these organisms (Raso et al., 1994, Scurrah et al., 2006a, Wang et al., 2009, Vercammen et al., 2012b).

Hydrodynamic Cavitation process optimization

Preliminary experiments were conducted to find out most effective rotor. The equipment comes with a 3 row rotor inside 4mm casing in default design, and the experiments were conducted for one pass at different flow rates on it. Later on same experiments were repeated with a 4 row rotor inside 6mm casing, which were found to be more effective and this rotor and stator combination was selected for the later study. Initial process design included hydrodynamic cavitation with a continuous recirculation as given in figure 2. Milk was dosed from the center of the flow cell and it came out from the top of the cell. This setting provided a flow rate of 200 L/h. Cavitator was adjusted to its maximum frequency for providing controlled hydrodynamic cavitation. Exposure time was calculated from equation 3.2, which is the amount of time for which milk was in contact within the radial holes of cavitator rotor during rotation. This was the multiple of total time of recirculation and total volume inside to caviator divided by the flow rate. Whereas, the total time was the time from inlet to outlet, which included inlet from balance tank, flowing inside pump cavitator through return pipes. After positive results in during continuous recirculation experiments, experiments were conducted with discrete recirculation (Figure 3) with 6 passes and later on were repeated at a temperature ≤ 72ºC.

Hydrodynamic Cavitation Treatment Outcome

Hydrodynamic cavitation after 6 passes resulted in significant inactivation of thermoduric vegetative cells of *Bacillus coagulans.* A count of 0.97 ± 0.03 log CFU/mL from an initial count of 4.05 ± 0.21 log CFU/mL was achieved by this treatment with continuous recirculation (Table 1). These results were equivalent to about 99.963% reduction in *Bacillus coagulans* vegetative cells (figure 4). The inoculated milk at 10°C was recirculated for 18 min equivalent to 6 passes or 6 cavitation effects (a total exposure time of 132 sec) with an average inlet and outlet temperature rise up to 68°C and 82°C respectively in the end. The results were verified by conducting trials up to 6 passes by passing milk from inlet tank through cavitator to the outlet tank. Upon comparison of rise in temperature to microbial cell reduction, no direct correlation of log reduction to temperature increase was observed (Figure 5). This signifies that the reduction was related to cavitation time and cavitation effect, irrespective of the differences in the rise in temperature from experiment to experiment.

The experiments were then conducted by passing milk through APV Cavitator to a lower end point temperature. In this case, the passes were continued until the temperature of product at outlet increased to 72°C. Temperature was monitored after each pass. The cavitated samples were microbiological analyzed using standard protocols and the data is presented in Table 2 and figure 6. The rise in temperature during each pass through the cavitator was also recorded. A count of 1.7 ± 0.08 log CFU/mL from an initial count of 5.64 ± 0.04 log CFU/mL was achieved

by this treatment. These results were equivalent to about 99.98% reduction in *Bacillus coagulans* vegetative cells. This percent reduction was similar to that of the results obtained with trials conducted at higher temperature. The results indicate that there was no correlation of variation in outlet temperature to log reduction. Denatured protein percentage was also measured and found going up from 75.75% to 78.4%. The increase is ~3% which could be due to either cavitation alone or cavitation in combination with heat treatment.

Hydrodynamic cavitation followed by Lab Pasteurization Treatment Outcome

Pasteurization alone was insufficient to significantly reduce vegetative cells of *Bacillus coagulans.* A count of 2.73 ± 0.20 log CFU/mL from an initial count of 4.05 ±0.21 log CFU/mL was achieved by lab pasteurization treatment at 63ºC for 30 min (Table 1). Hydrodynamic cavitation when combined with pasteurization resulted in a post treatment count of 0.67 ± 0.05 log CFU/mL. The combined treatment resulted in a greater inactivation of over 4 log CFU/mL, which was found statistically insignificant as compared to that of cavitation alone (Table 1). Combination of hydrodynamic cavitation and pasteurization significantly reduced bacterial cells up to 99.996% (Figure 4).

Comparison of hydrodynamic cavitation vs ultrasonication experiment

The process of hydrodynamic cavitation and ultrasonication is compared in table 3. Both these equipment, with their own equipment settings, were designed for pilot scale trials with relatively low flow rates (up to 200 liters per hour). In

comparison, the industrial setup may have flow rates up to thousands of liters per hour. Upon comparing the two techniques, hydrodynamic cavitation was found to be more efficient process, in terms microbial cell reduction, at higher flow volumes (200 Lit/h), as compared to ultrasonication (7.5 Lit/h). Some of the previous studies also suggested similar findings (Ashokkumar et al., 2011). Resulted achieved from hydrodynamic cavitation were also compared to that of ultrasonication. Hydrodynamic cavitation resulted in 99.99% reduction in bacterial cells of *Bacillus coagulans* after 6 passes at defined process parameters, whereas ultrasonication treatment resulted in 99.9% reduction in bacterial cells of *Bacillus coagulans* after 12 passes at defined process parameters (Figure 7). This signifies hydrodynamic cavitation as more effective technique in reduction of bacterial cells of *Bacillus coagulans* as compared to that of ultrasonication under the set of conditions tested.

Experiments on industrial samples

Industrial samples of pasteurized skim milk and skim milk concentrate were analyzed for their initial count and their species were identified by MALDI-TOF (Table 4).

In pasteurized skim milk samples, cavitation treatment resulted in temperature increase to 78.3°C, from initial temperature of 16.29°C. During this process, total bacterial count of 3.68 log CFU/mL was reduced to 1.44 log CFU/mL after 6-pass cavitation effect and further to Log 1.26 CFU/mL after 6-pass cavitation effect in combination with pasteurization. Total spore count of 2.26 log CFU/mL did not show any significant reduction after 6-pass cavitation effect, which remained

unchanged when cavitation was combined with pasteurization (Table 5). Cavitation of skim milk after 4 passes started showing reduction in total bacterial counts. Cavitation when combined with pasteurization resulted in over 2 log reduction after 6 passes. The possible reasons for observing a lower effectiveness of the cavitation for the commercial samples, as compared to that of proof of concept studies (Table 1), could be a result of the selection of thermally resistant strains due to their exposure and survival during prior heat treatments conducted at the manufacturing locations (Setlow, 2006a, Buehner, 2014). It is also possible that the population in pre-treated commercial milk samples could be predominantly spores. As the spores, due to their high hydrophobicity, become more resistant to heat and chemical treatments of manufacturing processes, they would also show greater resistance to any other inactivation processes. These resistant sporeformers adhere to the process surfaces and form heat resistant biofilms in pasteurizers from where they cross contaminate the products and become difficult to kill in pasteurized milk (Flint et al., 1997, Parkar et al., 2003, Gopal et al., 2015, Seale et al., 2015). These findings reported for the commercial samples are based only on one commercial pre-treated milk sample source, and would need further confirmation before drawing any conclusions.

Preliminary experiment using concentrate were also conducted. To evaluate the effect of hydrodynamic cavitation, milk concentrate samples were exposed to cavitation process under 3 different process conditions (Table 6). Temperature was monitored after each pass. The cavitated samples were microbiological analyzed

using standard protocols and the data is presented in Table 6 including the protein denaturation data before and after cavitation which was analyzed using Kjeldahl method to find out total casein and aggregated whey proteins. Cavitation was effective to kill most of the vegetative cells (99.65% reduction in Experiment 1 and 93.85% reduction in Experiment 2) in the concentrate, but showed lower reduction in spore counts (47.69% reduction in Experiment 1 and 48.67% reduction in Experiment 2). Experiment 3 with a single pass did not result in any significant reduction in sporeformers with initial and final counts at 3.04 ± 0.002 and 2.7 ± 0.03 respectively, and no reduction in spores. The probable for the lower effectiveness of hydrodynamic cavitation on skim milk concentrate compared to skim milk could be due to high total solids of the product (Piyasena et al., 2003). In addition, the denatured protein percentage started increasing when temperature exceeded beyond 78°C which could be due to increased time, temperature and cavitation effect on skim milk concentrate.

Effect of cavitation on the final sporeformer counts of skim milk powder

After 6 pass treatment, about 0.95 log CFU/mL survivors were left in milk, which finally resulted in 2.5 log CFU/g in the skim milk powder. In comparison, the bacterial counts were significantly higher at 5.0 log CFU/g in skim milk powder made from control (non-cavitated) samples. This study, though a simulation, helped to demonstrate the usefulness of including a hydrodynamic cavitation step in reducing the overall counts in skim milk powder by over 2 logs. The denatured

protein percentage was 81.12% before cavitation for untreated milk, which went up to 88.49% after cavitation.

Another set of experiment was conducted using industrial sample, where concentrate samples were cavitated and dried to see the impact of drying on spores and the final count in dried milk (Table 7). Cavitation followed by drying resulted in reduced counts by around 1 log CFU/mL.

These experiment supported that hydrodynamic cavitation helped reducing thermoduric organisms in skim milk and skim milk concentrate and the reduction was also visible in dried powders, though hydrodynamic cavitation treatment was found more effective in skim milk compared to that of skim milk concentrate. This could be due to high solid percentage in skim milk concentrate. The increase in denatured proteins can be controlled by controlling outlet cavitation temperature by adding jacketed tanks with coolant and also by adding PHE in the later passes. These results, however, suggested that applications of hydrodynamic cavitation can also be extended beyond skim milk to other dairy fluids.

CONCLUSIONS

A thermoduric strain of *Bacillus coagulans* was selected for studying the effectiveness of hydrodynamic cavitation treatment. Continuous hydrodynamic cavitation in combination with pasteurization was found as an effective method to reduce thermally resistant sporeformers such as *Bacillus coagulans* by 99.99%. The treatment was conducted for up to 6 passes, which was equivalent to 6 cavitation effects. These experiments were conducted at pilot scale, which demonstrates the

potential adaptability of this process at industrial scale. The outcome of this study showed promising results for application of cavitation caused by hydrodynamic cavitation in reduction of spoilage bacteria in milk. Extended trials with concentration of skim milk followed by drying resulted in 99.66% reduction in *Bacillus coagulans* cells. These results indicated that hydrodynamic cavitation is new technique which can be used by milk powder manufactures to reduce sporeformers in dried milks. Upon studying the effect on mixed population of bacterial cells and spores in industrial samples, a 99% reduction was observed in total bacterial count, whereas no significant reduction was observed in total spore count, which could be due to higher tolerance of thermally resistant organisms due to prior thermal processing in manufacturing lines. This high resistance may also be due to high tolerance developed in these organisms due to their exposure and survival at high temperature in industrial environment. No significant reduction of spores stated a need to further standardize this process to achieve desired effectiveness against spores.

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Table 1. Log counts of *Bacillus coagulans* cells in skim milk before and after hydrodynamic cavitation and pasteurization treatments alone as well as in combination with each other

	Mean	SE**
Initial	4.05^{a}	0.21
Pasteurization[2]	2.73^{b}	0.20
Cavitation[1]	0.97^{c}	0.03
Cavitation[1] + Pasteurization[2]	0.67^{c}	0.05

* Mean of n=6
[(a-c)] Mean without common superscripts are significantly different (P value <0.0001)
** SE= Standard Error
[1] Cavitation result after 6 pass
[2] Pasteurization at 63°C for 30 min

Table 2. Inlet and outlet temperature of skim milk along with Log count of *Bacillus coagulans* cells before hydrodynamic cavitation treatment and treatment after each pass till the temperature raised to 72°C.

Bacillus coagulans sporeformers

Parameters	Temp (°C)		Log Count
	Inlet	Outlet	
Control	8.0	-	$5.64^{a} \pm 0.04$
1 pass	20.5	35.1	$5.61^{ab} \pm 0.01$
2 pass	32.0	46.0	$5.42^{abc} \pm 0.008$
3 pass	40.7	55.8	$5.20^{bc} \pm 0.14$
4 pass	47.9	64.7	$5.40^{c} \pm 0.05$
5 pass	58.0	69.9	$3.11^{d} \pm 0.07$
6 pass*	-	72.0	$1.70^{e} \pm 0.08$

* Machine stopped immediately when outlet temperature reached 72°C

Table 3. Process conditions comparison between ultrasonication and hydrodynamic cavitation experiments and the comparison of results obtained from both the treatments.

		Units	Ultrasonication	Hydrodynamic Cavitation
Process conditions	Flow rate	L/h	7.5	200
	Residence time	min	2.2	16
	Back Pressure	kPa	50 psi	120
	Amplitude	µm	91.2	-
	Power	Watt	1000	-
	Number of passes	-	12	6
Results	Initial Log	CFU/mL	5	4
	Final Log after the treatments followed by pasteurization	CFU/mL	1.5	0.6
	% Reduction	%	99.9	99.99

Table 4. Species identified by MALDI-TOF from isolates obtained during plating of bacterial counts in skim milk and skim milk concentrate samples

Milk Samples	Isolates obtained during Total Counts	Isolates obtained during Spore Counts
Skim Milk	*Bacillus badius* *Bacillus subtilis*	*Bacillus pumilus* *Bacillus mojavensis* *Bacillus licheniformis*
Skim Milk Concentrate	*Bacillus licheniformis*	*Bacillus licheniformis*

Table 5. Total bacterial count and temperature increase after each cavitation effect alone and in combination with pasteurization in skim milk samples collected from manufacturing plant

Temperature during the process			Total Bacterial Counts (log CFU/mL) (Skim Milk)	
	Inlet Temp.(ºC)	Outlet Temp.(ºC)	Cavitation	Cavitation + Pasteurization
Control	16.29	34.7	3.26^{c} ± 0.04	2.39^{ef} ± 0.06
1	27.8	44.6	3.68^{a} ± 0.19	2.44^{e} ± 0.07
2	39.7	54	3.6^{a} ± 0.11	2.12^{g} ± 0.51
3	47.6	62	3.37^{b} ± 0.12	2.15^{fg} ± 0.06
4	55	69	2.77^{d} ± 0.05	2.01^{g} ± 0.09
5	60	73	2.48^{de} ± 0.07	1.39^{h} ± 0.40
6	66.9	78.3	1.44^{h} ± 0.04	1.26^{h} ± 0.10

Mean without common superscripts are significantly different

Table 6. Total plate count, Total spore counts, Denatured protein percentage, Temperature rise during hydrodynamic cavitation of skim milk concentrate samples collected from manufacturing plant after three different cavitation treatments

Experiment Number	Experiment Conditions	Total Plate Count (Avg., Log CFU/ml)		Total Spore Count (Avg., Log CFU/ml)		Temperature rise during Cavitation		$\frac{\text{Total Nitrogen} - \text{Non Casein Nitrogen}}{\text{Total Nitrogen}}\%$ (Kjeldahl method)	
		Initial count	Count after 6 Pass	Initial count	Count after 6 Pass	Initial	After 6 Pass	Initial	After 6 Pass
1	HC up to 6 pass with discrete recirculation with pre-heating upto 50°C	$4.8^{a} \pm 0.04$	$2.3^{d} \pm 0.03$	$2.1^{ef} \pm 0.02$	$1.8^{h} \pm 0.03$	45°C	94°C	78.04%	88.28%
2	HC up to 6 pass with continuous recirculation with no pre-heating	$3.11^{b} \pm 0.01$	$1.9^{gh} \pm 0.09$	$2.18^{e} \pm 0.05$	$1.89^{gh} \pm 0.08$	29°C	91°C	77.24%	88.08%
3	HC for 1 pass at lowest flow rate with no pre-heating	$3.04^{b} \pm 0.002$	$2.7^{c} \pm 0.03$	$2.04^{ef} \pm 0.04$	$2.0^{fg} \pm 0.04$	13°C	71.6°C	77.24%	79.22%

Each sample was plated in duplicates with 3 different dilutions. So total of 6 plates for each sample was counted, and mean counts are represented in Table

Mean without common superscripts are significantly different

Table 7. Total plate counts, Total spore counts, Denatured protein percentage before and after 6 pass hydrodynamic cavitation treatment of Skim milk concentrate and milk powder manufactured from it.

Experiment Number	Product	Total Plate Count (CFU/mL)		Total Spore Count (CFU/mL)		Denatured Protein Percentage	
		Initial	After 6 Pass	Initial	After 6 Pass	Initial	After 6 Pass
1	Skim Milk Concentrate	$4.11^{a} \pm 0.03$	$2.48^{d} \pm 0.02$	-	-	81.12%	88.49%
2	Milk Powder	$3.60^{b} \pm 0.05$	$2.42^{e} \pm 0.02$	$3.29^{c} \pm 0.05$	$2.27^{f} \pm 0.02$	-	-

Each sample was plated in duplicates
Mean without common superscripts are significantly different

Figure 1. The impeller with 4 row rotor and the cross- sectional view of APV Cavitator by SPX taken from APV Cavitator Technology for Ice Cream Mix Production Application sheet

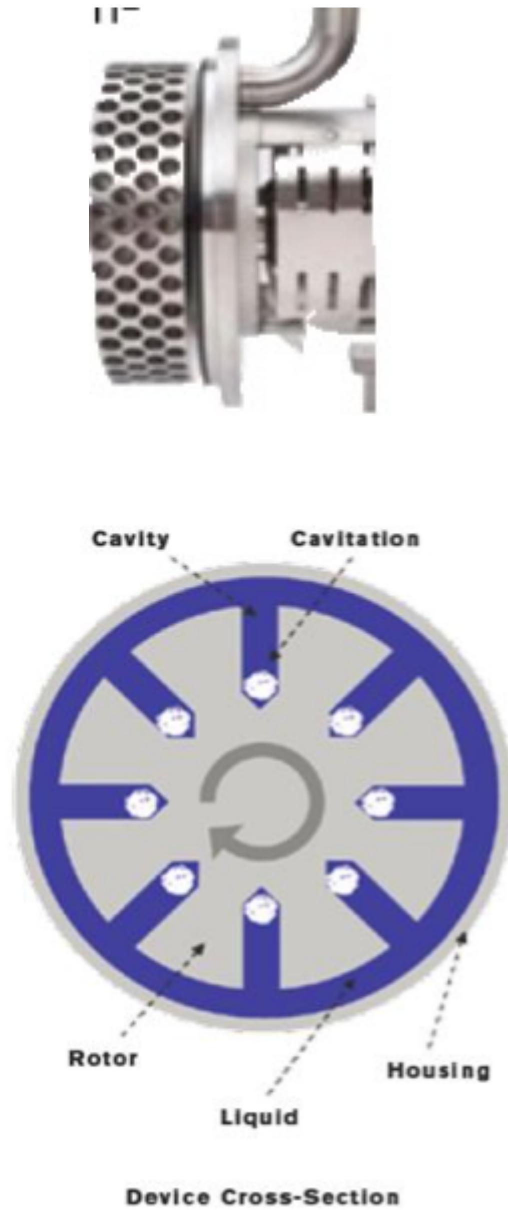


Figure 2. Process flow diagram of Hydrodynamic cavitation of Skim milk (Design 1) with in-line APV cavitator modified from APV Cavitator Technology for Ice Cream Mix Production Application sheet.

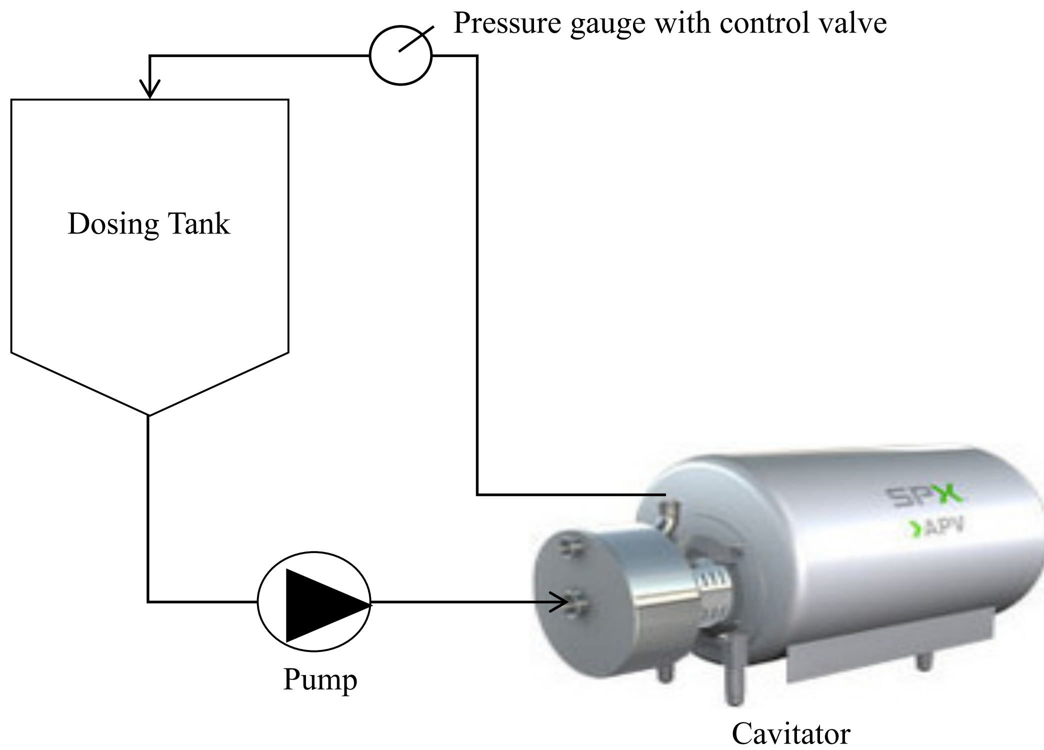


Figure 3. Process flow diagram of hydrodynamic cavitation with discrete recirculation of Skim milk (Design 2 and 3)

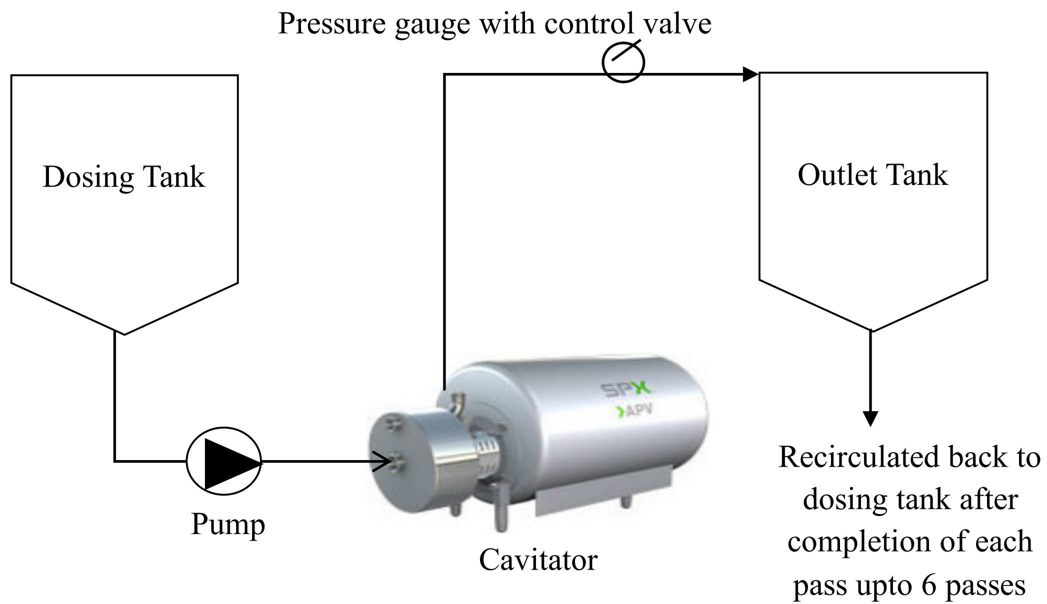
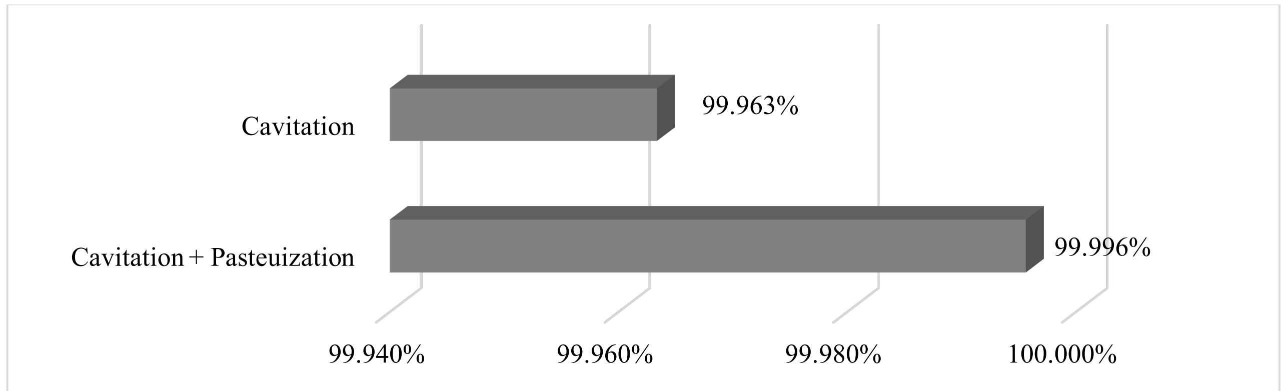
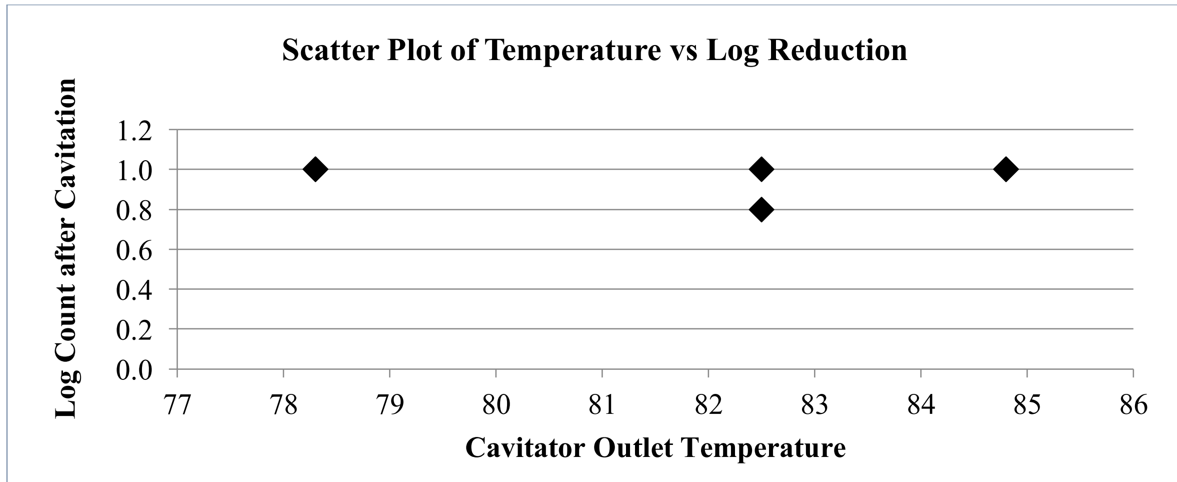


Figure 4. Percent inactivation in *B. coagulans* cells after hydrodynamic cavitation alone and after hydrodynamic cavitation in combination with lab pasteurization



Mean of n=6

Figure 5: Scatter plot of hydrodynamic cavitation outlet temperature to that of Log count after 6 pass treatment to evaluate any correlation in both



Results of 4 trials

Figure 6: Effect of hydrodynamic cavitation on reduction on Log count of *Bacillus coagulans* cells (—●—) after each pass vs rise in temperature of skim milk (—▲—) till the temperature raised to 72°C

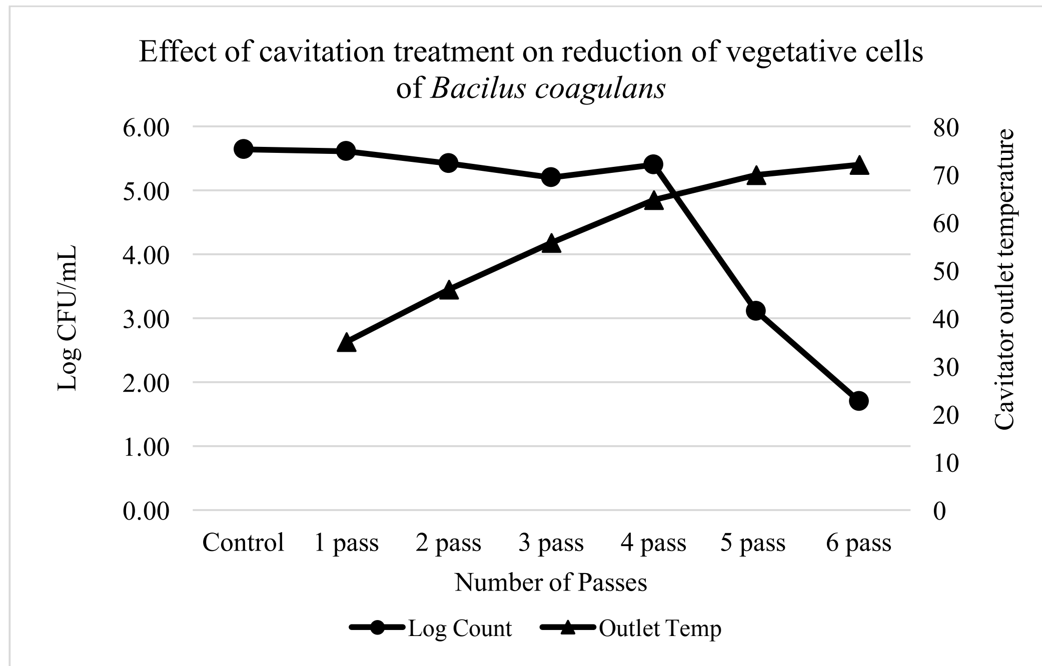
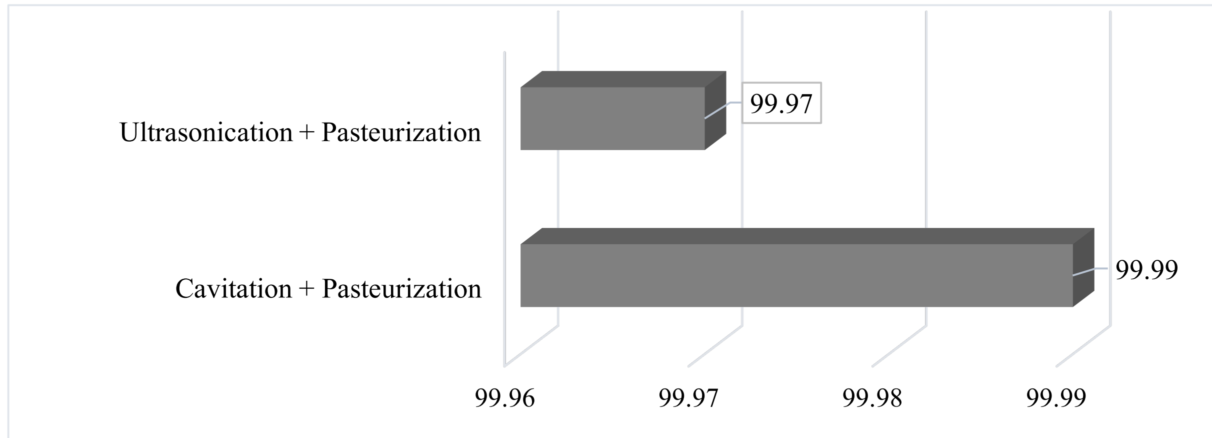


Figure 7. Percent inactivation in *B. coagulans* cells after ultrasonication combined with lab pasteurization compared to percent inactivation in *B. coagulans* cells after hydrodynamic cavitation in combination with lab pasteurization



Mean of n=6

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CHAPTER 4

EFFECT OF CONTINUOUS HYDRODYNAMIC CAVITATION ON THERMODURIC SPORES OF BACILLUS LICHENIFORMIS IN SKIM MILK

DIKSHI BAWA

ABSTRACT

Bacillus endospores can survive milk pasteurization, and later germinate and grow during further processing of milk. Their resistance to various physical and biochemical treatments, dormancy, ability to adhere to the surfaces of process equipment resulting in biofilm formation, and prompt germination under favorable conditions, make them important contaminants in dairy processes. The objective of this research was to investigate the effect of hydrodynamic cavitation in a continuous mode, and its combination with thermal treatment, to reduce *Bacillus* spores in skim milk. We hypothesized that cavitation would induce an effect similar to germination of spores and any post cavitation heat treatment would lead to their inactivation. Spores of *Bacillus licheniformis* (ATCC® 6634) were produced in lab by incubating on Brain Heart Infusion (BHI) agar plates for about 2 weeks, and were harvested from plates by washing and centrifugation. The resulting spores were inoculated in sterile skim milk to a level of ~2 log CFU/mL. Inoculated milk samples at 10°C were passed through APV Cavitator (SPX, Denmark) at 60 Hz frequency using 4 row rotor in 6mm housing at 200 L/h flow rate with a back pressure of 120 kPa. The milk

containing endospores was recirculated for 25 min (a total exposure time of 183 sec) with a temperature rise to 99°C. Samples were cooled and held for 3 hours at 30°C to let the germination process occur. Samples were then heated at treatment 1 (85°C for 15 min) and treatment 2 (80°C for 1 min), and were plated on BHI Agar. A preheating step was added before cavitation followed by treatment 2. Experiments were conducted as replicates of two, and were repeated thrice. Statistical significance of the data at $P < 0.05$ was determined using the SAS software. A significant difference was observed in the endospore counts after these treatments. Heat treatment alone did not result in inactivation of the spores. Whereas, cavitation with holding followed by heat treatment 1 resulted in inactivation of 97.59%, and preheating along with cavitation and holding followed by heat treatment 2 resulted in inactivation of 99.29%. In conclusion, it was evident that a combined process of cavitation, holding, and thermal treatments can be effectively used to inactivate low levels of thermoduric spores of *Bacillus licheniformis*.

KEY WORDS: spores, *Bacillus licheniformis,* milk, hydrodynamic cavitation

INTRODUCTION

Bacterial endospores are resistant to pasteurization and may affect quality of milk products (Khanal et al., 2014a, VanderKelen et al., 2016). The presence of bacilli in milk results in contamination of milk and dairy products and may lead to their shorter shelf life (De Jonghe et al., 2008). Under adverse conditions, such as insufficient nutrition or inappropriate growth environment, the spore formers get converted to spores. These spores then resist severe heat treatments, and others such as chemical treatments, and high pressure, which make them capable to survive under unfavorable conditions. *Bacillus* spores are capable to sense the availability of nutrients and respond by transforming back to a vegetative cell through germination (Driks, 2002, Seale et al., 2015). Processing at a very high temperature of 150ºC was also found insufficient to achieve complete inactivation of some spores (Lewis and Deeth, 2009a). Once they enter process environment, they are capable to form biofilms which are difficult to remove by various cleaning protocols (Parkar et al., 2003, Seale et al., 2008).

Bacillus licheniformis is one of the most prevalent sporeforming bacilli that can contaminate milk, which is capable to cause spoilage in milk powder during the product shelf life (Rückert et al., 2004, Trmčić et al., 2015). It is the most prevalent bacterial species identified in nonfat dry milk powders of Midwest origin (Buehner et al., 2014, Buehner et al., 2015). Similar observations were found in northeastern farms and manufacturing plants (Martin, 2014, Watterson et al., 2014). The optimal growth temperature for this bacteria is 30ºC with a growth range between 6ºC and a

higher temperature of 55ºC (Nissen et al., 2001, Burgess et al., 2010b). Some strains of *B. licheniformis* are reported as toxin producers, and some others have shown the ability to produce spoilage enzymes showing high proteolytic, lypolytic and glycolytic activity (Salkinoja-Salonen et al., 1999, De Jonghe et al., 2010, Carlin, 2011). Thermal treatments such as pasteurization and ultra-high treatment are not capable to kill *B. licheniformis* spores, as even a treatment of 77ºC for 15 s was found ineffective to kill these spores (Murphy et al., 1999). Another treatment using much higher temperature of extended shelf life products i.e. 127ºC for 5 s was also found unsuccessful in kill off all of these spores (Blake et al., 1995). All these reports established that heat treatments commonly used in dairy processing are not sufficient to kill spores of *B. licheniformis,* which indicate a need to explore non-thermal processes such as cavitation and combine them with existing thermal treatments to inactivate sporers. Various researchers also found survival of thermoduric organisms and their spores, which led to application of alternate methods in improve the inactivation rates of these organisms (Raso et al., 1994, Scurrah et al., 2006a, Wang et al., 2009, Vercammen et al., 2012b, Espejo et al., 2014). Researchers have used alternative techniques such as ultrasonication, UV-C light, Ultra High Pressure Homogenization, high pressure processing etc., to inactivate spores of *Bacillus* in milk and other food products (Daryaei and Balasubramaniam, 2013a, Gayán et al., 2013, Espejo et al., 2014, Khanal et al., 2014a).

Cavitation is one of the non-thermal techniques being explored in this study for its application in dairy industry. The treatment can be provided by ultrasonication (using sound waves) or by hydrodynamic cavitation (using a mechanical device). Milly et al., (2007) found applications of hydrodynamic cavitation to kill anarobic spores of *Clostridium sporogenes* in skim milk and aerobic spores of *Bacillus coagulans* in apple juice. Studies conducted by Khanal et al., (2014) used batch ultrasonication to significantly reduce spores of *Bacillus licheniformis* and *Bacillus coagulans.* The reduction went up when ultrasonication was combined with high heat treatment at 80ºC for 1 min. Similar success was achieved by Chouliara et al., (2010a) when they studied the effect of batch ultrasonication in combination with thermization, and ultrasonication in combination with pasteurization of milk on reduction of total viable counts.

The objective of this study was to investigate the impact of hydrodynamic cavitation in a continuous mode, combined with thermal treatment of skim milk spiked with thermally resistance *Bacillus* spores, and to optimize the process to achieve their maximum reduction. Previous research conducted in our lab proved the concept of cavitation produced by ultrasonication under static conditions as an effective technique in reduction of *Bacillus* spores in skim milk. Controlled Hydrodynamic Cavitation is an alternative to acoustic cavitation, which is a mechanically induced cavitation process. Bubble formation and rapid implosion of vapor-filled cavity during compression cycle in a fluid due to localized high-and low-pressure regions induced by mechanical means (Milly et al., 2007, Gogate,

2011). The mechanical rotor contains radial holes in it, and when it rotates these holes produce cavitation by spinning in a liquid chamber. The milk flows through this rotating impeller with holes where it is fed in from the center and comes out from the top (Figure 1). The rotating impeller with milk in it causes the pressure to fall below the vapor pressure of the medium due to velocity variations at a certain operating parameters resulting in formation of cavities The cavitation effect produce by hydrodynamic cavitation was found comparable to that of ultrasonication, however hydrodynamic cavitation was found more energy efficient and more compatible to handle large volumes (Gogate et al., 2006, Ashokkumar et al., 2011, Capocellia et al., 2014, Crudo et al., 2014).

MATERIAL METHODS

Culture Preparation and inoculation

Bacillus licheniformis (ATCC® 6634) was obtained from American Type Culture Collection center (ATCC). The culture was revived in 5 mL Brain Heart Infusion (BHI; Oxoid) broth using the procedure recommended by ATCC. The cells were pelleted by centrifugation (Beckman Coulter Avanti J-E centrifuge) at 4500 x g for 30 minutes. The pellets were washed thrice by using phosphate buffer saline (PBS) at pH 7.4 and by centrifugation at 4500 x g for 15 minutes. Final washed suspension was stored in 1.8 mL cryo vials containing beads and glycerol at -80°C in deep freezer (NuAire ultralow freezer) for entire duration of experiments. The organism was studied for its growth curve and generation time by using BHI Broth

and their number was counted by plating done using BHI Agar. Optical density of the turbid broth was also measured by spectrophotometer (Thermo scientific Spectronuc 200) (Widdel, 2007). Colonies from BHI agar plates were taken out and stained using spore staining technique to monitor sporulation percent (Hamouda et al., 2002, Buehner, 2014).

Spores were produced in lab by incubating on Brain Heart Infusion (BHI) agar plates at 37ºC for about 2 weeks, and were harvested from plates by washing with 10 mL of sterile distilled water and transferring to 50 mL centrifuge tube for centrifugation. Centrifugation was done at 4500 x g for 30 minutes following by washing with distilled water. The washed pallet was then suspended in 10 mL distilled water. The vegetative cells in spore suspension were inactivated by heating at 85°C for 15 min. The resulting spores were stored at -18ºC for using during further experiments. These spores were then inoculated in sterile skim milk to a level of log 2 CFU/mL.

Hydrodynamic cavitation and preheating treatment:

A 10 L of sterile skim milk was inoculated with bacterial spores at ~2 log CFU/mL. The spiked milk was then poured in a sanitized stainless steel tank for cavitation treatment. It was passed through APV Cavitator (SPX, Denmark) at 60 Hz frequency using 4 row rotor in 6mm housing at 200 L/h flow rate with a backpressure of 120 kPa (Figure 2). Design 1 created for sporeformer trials was used with a continuous recirculation. Trials started from 18 min recirculation (~ 6 passes)

and were recirculated for 25 min (~8 passes). Samples were taken for further heat treatment.

Design 2 was created by adding a preheating step before cavitation, and the samples were cavitated similar to design 1. Samples were taken for thermal treatment of 80°C for 1 min. A validated cleaning in place (CIP) procedure was followed for effective cleaning and sanitation the equipment.

Spore Germination Studies

Spores of *Bacillus licheniformis* were inoculated in skim milk and then treated with hydrodynamic cavitation. Sample were drawn to study the impact of cavitation on germination of spores that survived the process. Samples were cooled and held for 3 hours at 30°C to let the germination process occur by replicating the germination process as previously standardized by Khanal et al., (2014) in our lab.

High Heat Treatment

A control sample was drawn before the treatment, a cavitated sample was drawn after cavitation treatment, and a germinated sample was drawn from cavitation followed by holding. A 3 mL of each of these samples was transferred to a sterile test tube with sterile threaded cap. Tubes were immersed in water bath preset at desired temperatures of treatment 1 and treatment 2 independently. A preheating step was introduced before cavitation followed by treatment 2. Samples were then thermally treated at 85°C for 15 min considered as treatment 1 (T1) and at 80°C for 1 min considered as treatment 2 (T2) before plating. The time and temperature was

monitored to achieve desired heating. The samples were then removed from hot water bath and kept in chilled water beaker till the temperature reached <5°C. These samples were used for plating to calculate spore survivors after these treatments.

Enumeration of spores

All Control (before treatment) and heat treated milk samples were plated on BHI agar to calculate the survivors. Dilutions were made using 9 mL PBS solution. All counts were taken using standard pour plate technique (Laird et al., 2004). Plates were incubated inverted for 24 hours at 37°C. Colonies were counted as CFU/mL as per equation 2.3.

$$N = \frac{\Sigma C}{\{(n1 \times 1)+(n2 \times 2)\}d} \quad (4.1)$$

N = CFU/mL

ΣC = Sum of colonies on all counted plates

n1= Number of plates of lower dilution

n2= Number of plates of higher dilution

d= dilution level of n1 (lower count)

Percentage reduction of vegetative cell counts after each treatment was calculated as per equation 2.4.

$$\%\ \text{Inactivation} = \frac{\text{Counts before treatment} - \text{Counts after treatment}}{\text{Counts before Treatment}} \times 100 \quad (4.2)$$

Scanning Electron Microscopy

For Scanning Election Microscopic (SEM), purified spores were suspended in sterile distilled water, cavitated and heat treated. Treated suspensions were dried on a glass slide, coated with 5nm gold layer using sputtering technique at South Dakota State University department of electrical engineering. Slide were then visualized at different magnifications under Hitachi Scanning Electron Microscope.

Statistical Analysis

Statistical significance of the data at $P < 0.05$ was determined by ANOVA using the SAS 9.2. MS excel was used to calculated mean, standard deviation and standard error, and to generate graphical representation of data.

RESULTS AND DISCUSSIONS

Bacillus licheniformis (ATCC® 6643) inoculation studies

Spores of *Bacillus licheniformis* (ATCC® 6643) were taken out from -18ºC and were thawed to reach room temperature. The suspension was vortexed and inoculated in skim milk ~2 log CFU/mL in milk. The inoculated milk was studied for survival of this bacteria against pasteurization treatment at 63ºC for 30 min and for high heat treatments at 85ºC for 15 min and at 80ºC for 1 min before beginning hydrodynamic cavitation treatment. Given in table 1 and table 2 are the results after survival at high heat treatment.

High heat treatment was not able to result in any reduction of these cells (Tables 1 and 2). These findings were similar to previous research work conducted in our lab (Buehner, 2014, Khanal et al., 2014a).

Hydrodynamic Cavitation and heat treatment of spores

Preliminary experiments were conducted as per design 1 with 4 row rotor inside 6mm casing for 18 min recirculation treatment to replicate the process conditions for sporeformers as per figure 2. The recirculation time was gradually increased till the temperature reached to 99°C. Samples were drawn for further treatment i.e. a) heating by treatment 1 (85ºC for 15 min), b) holding at 30ºC for 3 hours to allow time for spores to germinate, so they can subsequently be inactivated by treatment 1.

A count of 1.24 ± 0.05 log CFU/mL from an initial count of 2.2 ± 0.03 log CFU/mL was achieved by this treatment with continuous recirculation (Table 1). The cavitation treatment when combined with heating at 85°C for 15 min resulted in further reduction to 0.88 ± 0.02 log CFU/mL. The reduction was increased with holding step addition with a final log count of 0.60 ± 0.03 log CFU/mL. Overall, hydrodynamic cavitation when combined with heating (85°C for 15 min) resulted in a greater inactivation of 97.59%, which was found effective to reduce the counts whereas heating alone (85°C for 15 min) was ineffective to reduce these spores (Figure 3).

On the basis of above trials, the heat treatment of 85°C for 15 min was not found to be feasible for implementation at milk powder manufacturing plants.

Therefore, design 2 was studied, which was more similar to process design of powder manufacturing, where usually there is a preheater placed before evaporator and milk is preheated before entering evaporator 1st effect (Murphy et al., 1999, Scott et al., 2007a, Walstra, 2013). The process resulted in a final spore count of 0.83 ± 0.04 log CFU/mL from an initial count of 2.36 ± 0.07 log CFU/mL that was achieved by this treatment with continuous recirculation (Table 2). This treatment when combined with heating at 80°C for 1 min resulted in further reduction of spores to 0.53 ± 0.02 log CFU/mL. The reduction was further increased with the addition of holding step prior to heating with a final log spore count of 0.21 ± 0.01 log CFU/mL. Therefore, preheating combined with cavitation followed by holding and subsequent heating (80°C for 1 min) lead to a reduction in spore counts by 99.29% (Figure 3). In addition to that, the SEM images (at 14000x) of spores after each of these treatments also provided the evidence of spore destruction due to cavitation alone or cavitation followed by heating. Deformed spore structures were observed after cavitation, a greater deformation of spores was observed in cavitated and heated samples that could potentially be an inactivation. There could be some germination of spores during holding, these germinated cells got disintegrated during heating. The cell fragments were also observed in cavitated and heated sample, when visualized under SEM (Figure 4). The results when compared with studies conducted by Khanal et al. 2014 using acoustic cavitation in combination with pasteurization, where they found up to 4 log CFU/mL reduction in *Bacillus licheniformis* spores. They also studied that replacing high heat treatment of 80ºC for 1 min with pasteurization at 63ºC for

30 min was found more effective in reduction of spores. Other studies conducted by Milly et al. 2007, found upto a 2.84 log CFU/mL reduction of anarobic spores of *Clostridium sporogenes* in skim milk and upto a 3.10 log CFU/mL reduction of aerobic spores of *Bacillus coagulans* in apple juice. Upon using other non-thermal techniques such as ultra high pressure homogenization on *Bacillus* spore reduction, a ~5 log CFU/mL decrease was observed (Espejo et al., 2014). A UV-C light treatment resulted in upto ~4.05 log CFU/mL reduction in *Bacillus licheniformis* spores (Gayán et al., 2013). A pulsed electric field treatment was found triggering *Bacillus subtilis* spore germination followed by heating resulting in inactivation of germinated spores (Shin et al., 2010). Sasagawa et al., 2006, found that a combination of more then one non-thermal technique was found more effective than using a single technique alone. Upto a ~7 log CFU/mL reduction in *Bacillus* spores when HHP and PEF treatments were used in combination. This was found more effective than using either of these treatment alone (Sasagawa et al., 2006). Similarly a combined pressure and heat treatment resulted in upto a ~4.4 log CFU/mL reduction in *Bacillus licheniformis* spores which was higher than any of these treatments alone (Scurrah et al., 2006a). In more recent studies a similar log reduction in *Bacillus* spores was achieved PEF treatment was combined with heating (Siemer et al., 2014).

CONCLUSIONS

Thermoduric spores of *Bacillus* species i.e. *Bacillus licheniformis* are one of the most common species of spores present in milk. These spores were selected for studying the effectiveness of hydrodynamic cavitation treatment. A continuous cavitation treatment combined with holding step and thermal treatment was found effective to reduce thermally resistant spore such as *B. licheniformis* by over log 2 CFU/mL. These experiments were conducted at pilot scale, which would increase the adaptability of this process at industrial scale. The outcome of this study shows promising results for application of cavitation obtained by hydrodynamic cavitation combined with thermal treatment in reduction of spore of common spoilage bacteria in skim milk.

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Table 1. Log CFU/mL of *B. licheniformis* spores before and after cavitation and heating at 85ºC for 15 min (T1)

Step	Mean*	SE**
Initial Count	2.22[a]	0.03
Count after heating	2.17[a]	0.03
Cavitation	1.24[b]	0.05
Cavitation + heating	0.88[c]	0.02
Cavitation + holding + heating	0.60[d]	0.03

* Mean of n=6
[(a-d)] Means in each table without common superscripts are significantly different (*P*-value <0.05)
** SE= Standard Error

Table 2. Log CFU/mL of *B. licheniformis* spores before and after cavitation and heating at 80°C for 1 min (T2)

Step	Mean*	SE**
Initial Count	2.36^{a}	0.07
Count after heating	2.22^{a}	0.08
Preheating[1] + Cavitation	0.83^{b}	0.04
Preheating[1] + Cavitation + heating	0.53^{c}	0.02
Preheating[1] + Cavitation + holding + heating	0.21^{d}	0.01

* Mean of n=6
$^{(a-d)}$ Means in each table without common superscripts are significantly different (*P*-value <0.05)
** SE= Standard Error
[1] Preheating upto 65°C

Figure 1. APV Cavitator by SPX (SPX APV Cavitator Technology in Food and Beverage Processing)

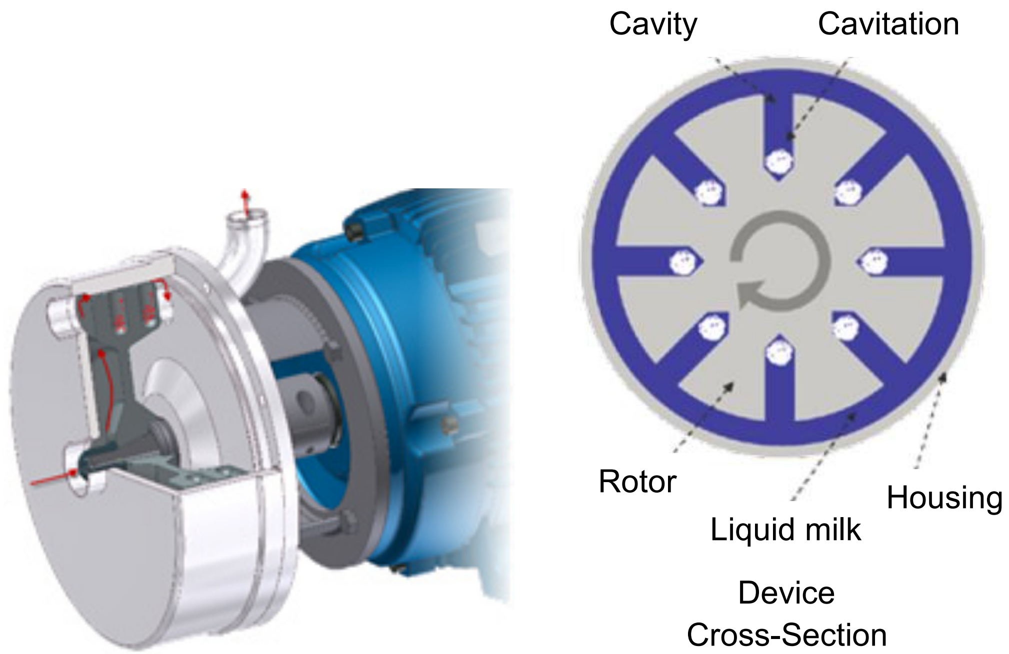


Figure 2. Process flow diagram of Cavitation of Skim milk

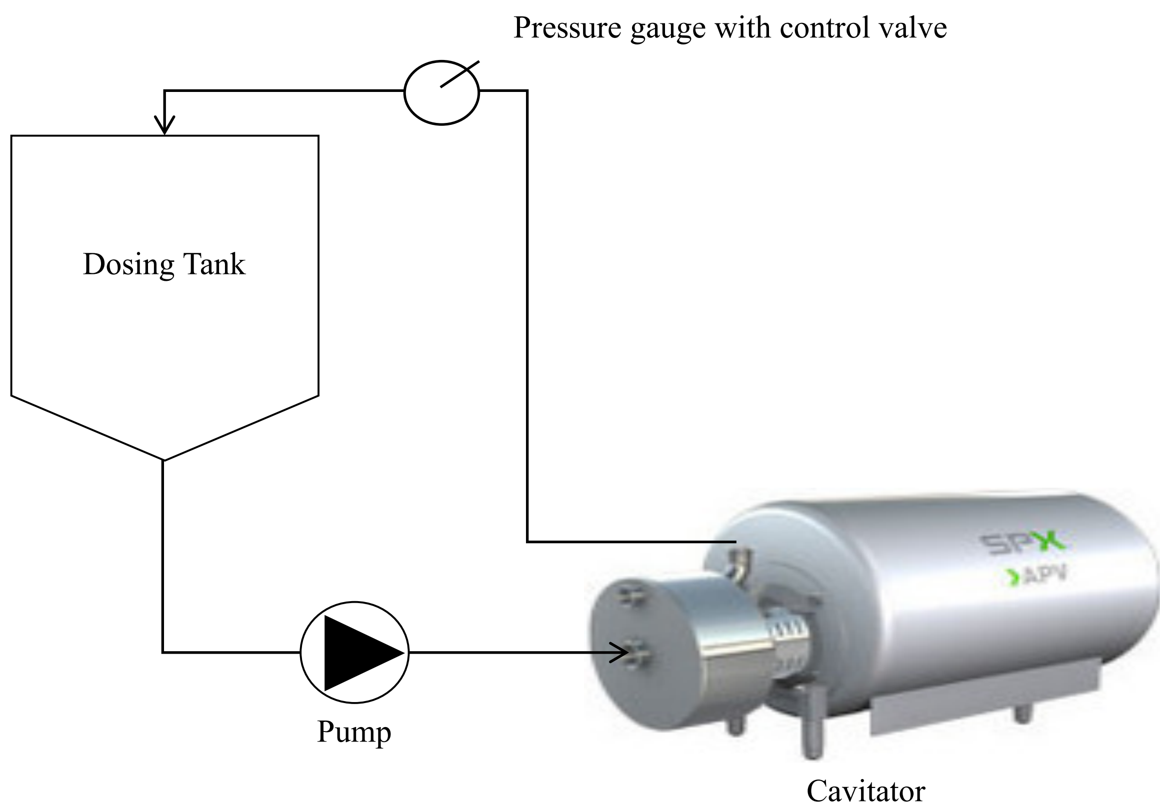


Figure 3. Percent inactivation in *B. licheniformis* spores after treatment 1 (T1) and treatment 2 (T2)

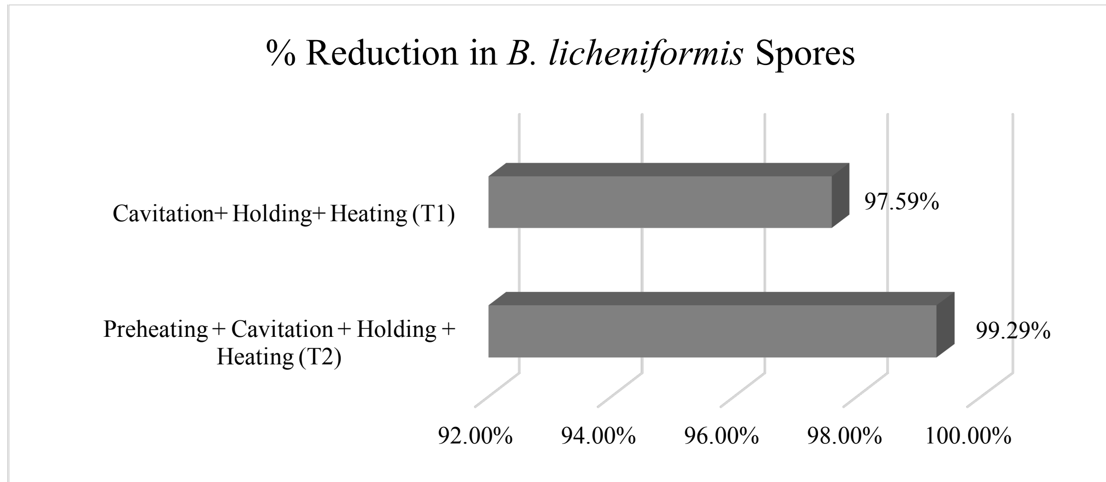
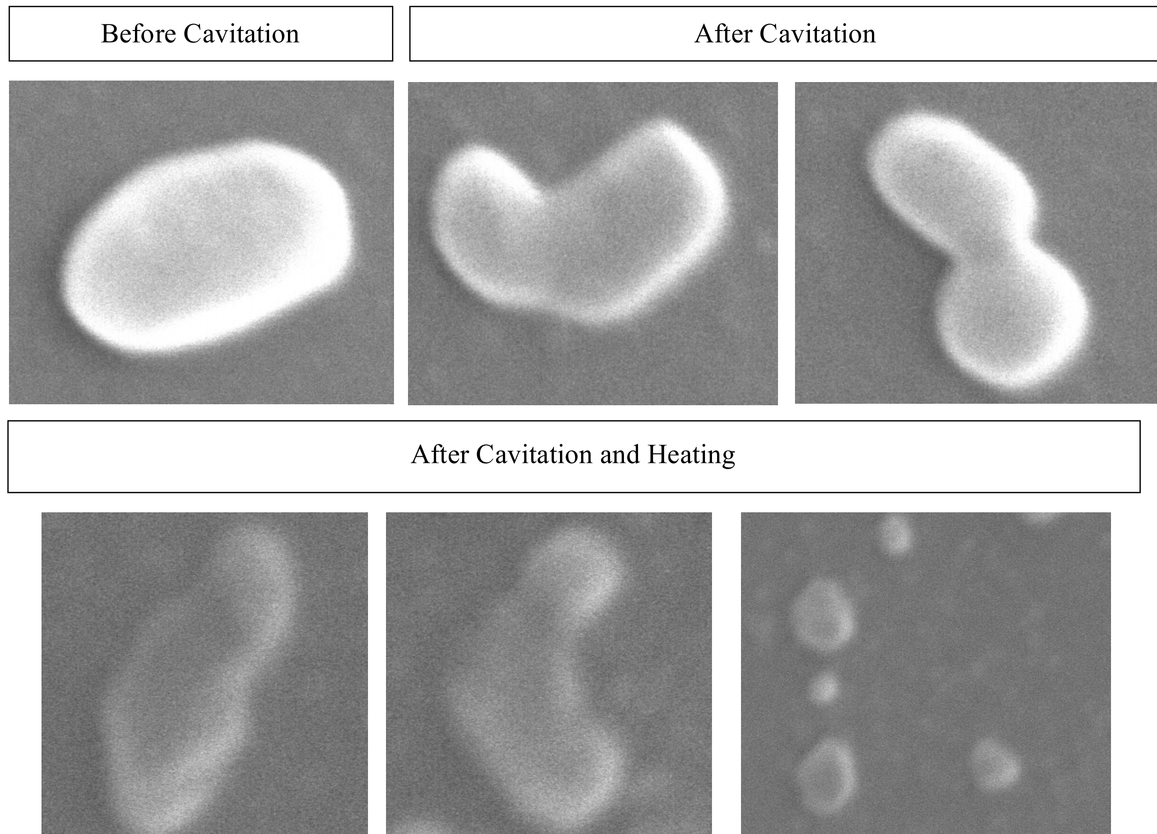


Figure 4. SEM images (taken at 14,000x magnification) of spores of *B. licheniformis*



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OVERALL SUMMARY AND CONCLUSIONS

The research summarized in this thesis explains the application of the cavitation phenomenon produced by ultrasonication or hydrodynamic cavitation to kill thermoduric aerobic sporeformers of *Bacillus* spp. in skim milk, and skim milk concentrate. Thermal processes such as pasteurization and higher heat treatment are capable to kill pathogens in milk. However, heat resistant sporeformers and their spores can survive these thermal treatments, as well as, other adverse process conditions and cause cross contamination of milk and subsequently dairy products resulting in product quality and safety issues. Raw milk gets contaminated from surrounding environment and a bacterial load of >10^5 CFU/mL is an indicator of poor sanitary conditions. Various *Bacillus* species such as *Bacillus licheniformis, Bacillus coagulans, Anoxybacillus flavithermus, Geobacillus stearothermophilus* and *Bacillus sporothermodurans* are common contaminants of milk processes and end products. Plate heat exchangers, pasteurizer, preheaters, and evaporator are the main sites where these organisms can grow and multiply. The spoilage caused by these species is not limited to milk, it can also pass through the process barriers of heat treatments and can contaminate final product that are pasteurized, UHT or sterilized milk or any subsequent dairy products such as yogurt, cheese and milk powders. All this makes these organisms critical for dairy industry.

Sporeformers of *Bacillus coagulans* were investigated for their resistance against pasteurization. A count of 3.88 ± 0.13 log CFU/mL from an initial count of

5.20 ± 0.09 log CFU/mL was achieved by lab pasteurization treatment. Similarly spores of *Bacillus licheniformis* were high heat treated at 85ºC for 15 min and the treatment resulted in no reduction at all. Therefore, ultrasonication and hydrodynamic cavitation were applied to increase the effectiveness of heat treatment. Previous studies in our lab tested ultrasonication under static condition and found it effective in reduction of bacilli in milk. In continuation to that, the ultrasonication process used in current research was designed to study the effectiveness of ultrasonicator in a continuous mode. For that, a dosing tank and a dosing pump was connected to with a Hielscher USA, Inc 1000 W UIP1000hd ultrasonicator connected to an outlet tank for collection of milk after one pass. Milk was then transferred back to dosing tank for another pass. Ultrasonication after 12 passes resulted in up to 92% reduction in *Bacillus coagulans* vegetative cells. Further, a combination of ultrasonication and pasteurization significantly enhanced the reduction of bacterial cells to 99.98%. The experiments were conducted at pilot scale ultrasonicator and can be adopted at industrial scale. To increase the efficacy of ultrasonication during scale up, various high power equipment such as Hielscher ultrasound UIP 16000 with 16000 W of power supply and sonication units of 7 x UIP1000hdT can also be used. The outcome of this study showed promising results for application of ultrasonication in upto 3 log CFU/mL reduction of spoilage bacteria in milk.

Hydrodynamic cavitation was another cavitation process used in current research to study its effectiveness in a continuous mode to kill heat resistant

sporeformers and spores of *Bacillus* species in milk and milk products. An APV cavitator from SPX flow technology, was connected to a dosing pump and dosing tank to dose inoculated skim milk. The milk was then continuously recirculated for defined time durations. In experiments with sporeformers, a discrete recirculation was also designed by connecting cavitator outlet to an outlet tank for milk collection after each pass. The results revealed that 6 passes alone and in combination with pasteurization were found effective in inactivating thermally resistant vegetative cells of *Bacillus coagulans* by 99.963% and 99.996% respectively. Their progression was also studied through skim milk powder manufacturing steps under pilot scale, using concentration and drying processes. The treatment resulted in over 99.66% reduction in vegetative cells of *Bacillus coagulans* in skim milk powder. The reason of obtaining lower reduction at the skim milk powder stage to that of skim milk was attributed to be due to the possible multiplication of survivors during the milk concentration process.

The experiments related to *Bacillus licheniformis* spores inactivation were also conducted using continuous recirculation experiments using the hydrodynamic cavitator. Inoculated skim milk samples were cavitated, and held for germination prior to heat treatment at high heating temperatures to kill damaged spores and germinated cells. Cavitation along with holding for 3 hours at 30°C, followed by heat treatment of 85°C for 15 min, resulted in inactivation of 97.59%. On the other hand, preheating at 65°C followed by cavitation, holding and heat treatment of 80°C for 1 min resulted in inactivation of 99.29%. The combined process of cavitation, holding,

and thermal treatments can thus be effectively used to inactivate thermoduric spores of *Bacillus licheniformis*.

Our studies indicated that non-thermal techniques, such as cavitation, when combined with heat treatments would increase their efficacy in inactivation of thermally resistant sporeformers and spores of bacilli in skim milk. The research also demonstrated the application of ultrasonication and hydrodynamic cavitation in a continuous process to inactivate these organisms. Cavitated milk when pasteurized at a temperature of 63°C for 30 min was found to have significantly reduced load of thermoduric sporeformers such as *Bacillus coagulans*. This implies that cavitator can be added before pasteurization process in the processing line. This will enhance the thermal inactivation of the damaged cells that survived the cavitation process. This will not only result in low bacterial load in the processed milk, but will also cause reduced biofilms in pasteurizers due to less bacterial counts in milk. Furthermore, preheated milk when cavitated and heated at evaporation temperature of 80°C for 1 min showed significant reduction in *Bacillus licheniformis* spores. Therefore, in milk powder manufacturing line, cavitator can be added after preheater and before 1st effect evaporator to reduce spore counts and biofilm formation in evaporator. Overall, the research generated new information on the impact of cavitation to produce skim milk and milk powder with low bacterial count. This is also likely to prevent biofilm formation in heating exchangers and evaporators in the process line. This research work forms the basis for future studies where cavitation can be applied under various process conditions with varying range of temperature and passes to

reduce bacterial load in dairy ingredients such as whey protein concentrate, milk protein concentrate, lactose, filtration retentate etc., and in dairy products such as UHT milk, yogurt, cheese etc.